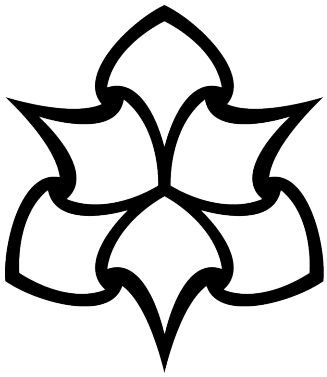

Building physiological reserve in immobilisation: does nutritional supplementation work?

EMMA LOUISE BOSTOCK

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Department of Exercise and Sport Science
Manchester Metropolitan University

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Publications

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Bostock, E.L., Morse, C.I., Winwood, K., McEwan, I. & Onambélé, G.L., (2016). Omega-3 fatty acids and vitamin D in immobilisation: Part B- Modulation of muscle functional, vascular and electromyographic profiles. *The journal of nutrition, health & aging*, pp 1-8
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List of abbreviations

Abbreviation	Definition
ACSA	Anatomical cross sectional area
BMC	Bone mineral content
BMD	Bone mineral density
CK	Creatine kinase
CSA	Cross sectional area
CV	Coefficient of variation
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DXA	Dual-energy x-ray absorptiometry
EAA	Essential amino acid
ELISA	Enzyme-linked immunosorbent assay
EMG	Electromyography
EPA	Eicosapentaenoic acid
FAK	Focal adhesion kinase
FbD	Flow by diameter
FFT	Fast Fourier transform
GLUT4	Glucose transporter type 4
HR	Heart rate
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin 6
IL-10	Interleukin 10
MAFbx	Muscle atrophy f-box
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MuRF-1	Muscle ring finger 1
MVC	Maximal voluntary contraction
ng/mL	Nanogram per millilitre
PCSA	Physiological cross sectional area
pg/mL	Picogram per millilitre
PLA	Placebo group
pQCT	Peripheral quantitative computed tomography
RI	Resistance index
RNA	Ribonucleic acid
ROS	Reactive oxygen species
TNF-α	Tumour necrosis factor alpha
ULLS	Unilateral lower limb suspension
U/L	Unit/Litre
VDR	Vitamin D receptor
Vit-D	Vitamin D group
ω-3	Omega-3
µg	Micrograms
µl	Microlitres

Abstract

Introduction: Disuse models, such as limb immobilisation, result in profound changes in skeletal muscle morphology and function. Exercise prescription would be the recommended intervention to prevent immobilisation-induced atrophy and declines in maximal voluntary strength. Nutritional supplementation may stand as a viable intervention to combat muscle atrophy with disuse, when exercise is an unpractical therapeutic option.

Aims: To (1) investigate the multifactorial effects of short-term upper limb sling immobilisation and (2) determine whether three potential protein-sparing modulators (essential amino acids (EAA), omega-3 (ω-3) and vitamin D) would attenuate the anticipated deleterious effects of immobilisation.

Methods: Measures of muscle and sub-cutaneous adipose thickness (Brightness mode ultrasonography), body composition (dual-energy x-ray absorptiometry), arm girth (anthropometry), isometric and isokinetic elbow torque (dynamometry), co-contraction (electromyography (EMG)), muscle fatigability (dynamometry and EMG), arterial blood flow (Doppler ultrasound) and endocrine profile (enzyme-linked immunosorbent assay and colorimetry), were taken before and after arm immobilisation in a mixed sex population. Supplementation of EAA (n = 9 vs. placebo n = 7) during three weeks of immobilisation, ω-3 (n = 8) or vitamin D (n = 8) during two weeks of immobilisation (placebo n = 8) and EAA for two weeks pre-immobilisation (n = 5 vs. placebo n = 5).

Main findings: Immobilisation resulted in decreases in muscle thickness, arm girth, lean mass, isometric and isokinetic elbow torque, and an increase in sub-cutaneous adipose thickness. Muscle fatigability, resting arterial blood flow, EMG co-contraction and endocrine profile were unchanged. At the current dosage ω-3

supplementation only attenuated the increase in sub-cutaneous adipose thickness. Despite some trends, neither ω-3 nor vitamin D supplementation attenuated any other parameters. EAA supplementation during immobilisation impacted positively on the immobilisation-induced changes in the structural and functional characteristic of the remaining muscle. EAA supplementation before immobilisation did not attenuate the immobilisation-induced changes in muscle structure and function.

Conclusion: Although EAA supplementation only showed some benefit to muscle size and function with immobilisation, it was confirmed that the sling immobilisation model used in the thesis, is a suitable model for observing the effects of relatively short-term immobilisation. The findings of the thesis are relevant to both sporting (e.g. off-season detraining modulation) as well as clinical (e.g. injury/illness induced short-term immobilisation/bed rest) populations. This relatively short-term sling immobilisation provides a model to be used to assess other supplements and treatments in future studies. The modest effect of supplementation suggests further research into either: a) more at risk populations (e.g. injury or ageing); b) larger doses of these supplements.

Chapter 1: Thesis introduction and literature review

This Chapter appears in publication as: Bostock, E. L., Morse, C. I., Winwood, K., McEwan, I., and Onambélé-Pearson, G. L. (2013) 'Hypo-activity induced skeletal muscle atrophy and potential nutritional interventions: A review.' World Journal of Translational Medicine, 2(3), pp. 36-48.

Thesis introduction

Skeletal muscle is one of the most adaptable tissues in the body, and as such, it is capable of altering its structure in response to different levels of physical activity. Prolonged reductions in muscle activity and mechanical loading result in many physiological adaptations in skeletal muscle size and function (Kawakami et al., 2001; LeBlanc et al., 1992; Veldhuizen et al., 1993; White et al., 1984). Muscle atrophy (decrease in muscle mass) is seen during reduced activity (e.g. sedentary behaviour, de-training) (Andersen and Aagaard, 2000; Hakkinen et al., 2000; Hortobagyi et al., 1993; Narici et al., 1989) or disuse models (e.g. immobilisation, bed-rest) (Akima et al., 2000; Grosset and Onambele-Pearson, 2008; Kawakami et al., 2001; Veldhuizen et al., 1993). It is evident that the degree of muscle atrophy is not constant across muscle groups or hypo-activity models (Convertino, Doerr, Mathes, et al., 1989; LeBlanc et al., 1992; Veldhuizen et al., 1993; Yue et al., 1997).

Simply reducing normal levels of activity can be classed as the first stage of disuse. Decrements in muscle mass and strength have been documented in trained humans undergoing de-training (Andersen and Aagaard, 2000; Colliander and Tesch, 1992; Hakkinen et al., 2000; Hakkinen and Komi, 1983; Hortobagyi et al., 1993; Houston et al., 1983; Narici et al., 1989). Bed-rest conditions result in the removal of normal weight-bearing forces acting on the bones of the lower limbs in the vertical position and a decrease in number and/or magnitude of muscle contractions, particularly in the postural musculature. During bed-rest, muscular contraction is still possible although it is limited and the muscular force required for producing movement is very much diminished, once ground reaction forces are removed. A more rigid immobilisation can be achieved by casting a limb, resulting in more rapid decrements in muscle mass than does bed-rest alone. The final

method of hypo-activity commonly reported in the literature, is that of unilateral lower limb suspension (ULLS), a method of reducing habitual activity whilst causing lesser degree of inconvenience to the participants.

The aim of this chapter is to assess the varying impact of different hypo-activity models on the skeletal muscle system. This is broken down into the effects of hypo-activity on muscle morphology, muscle strength and muscle fatigability. In order to provide some homogeneity in the results based on the variable duration of the hypo-activity, values are presented per week and where relevant the duration of the hypo-activity is provided in parenthesis. Exercise is not always a practical prescription, even when it would be recommendable, to individual's under-going immobilisation or bed-rest after trauma or illness, due to the presence of contraindications for exercise such as pain, immobilisation in a cast etc. In some cases of hypo-activity, isometric and static contractions are still possible but not in all cases, for example with muscle tears. Thus, other interventions are required to attenuate losses in muscle mass and function. Therefore, this chapter will also discuss potential nutritional interventions for preventing the loss of muscle mass/function seen with hypo-activity, where increased physical activity is not combined with the nutritional treatment. Studies were found using search terms 'bed-rest and atrophy' and 'immobilisation and atrophy' in PubMed. However, this returned over 1400 hits. To focus the search criteria and make it relevant to the population to be examined in subsequent chapters, only data on healthy humans were selected through the inclusion of the 'human' and 'clinical trial' filters in the PubMed search. This resulted in 98 studies, suitable for inclusion in the present chapter. Some hand searching and an additional search with the term 'disuse' was completed to insure no relevant texts were ignored.

Literature review

1.1. Muscle morphology

1.1.1. Muscle anatomical cross sectional area

Anatomical cross sectional area (ACSA) is the cross-sectional area of the muscle at right angles to its longitudinal axis. Muscle ACSA is a major determinant of maximal voluntary contraction (MVC) torque (Bamman et al., 2000; Narici et al., 1992) and hypo-activity models have been shown to result in the decrease in this parameter (Figure 1.1.). Periods of detraining (24 weeks) have resulted in a decrease in ACSA of the quadriceps (Hakkinen et al., 2000). Likewise, Narici et al. (1989) reported decreases in leg ACSA (~0.7 %·wk^{-1}) in response to 40 days de-training.

Stricter hypo-activity models result in greater decreases in muscle ACSA. Following 30 days bed-rest Convertino et al. (1989) reported decreases in ACSA of the calf (~1.1 %·wk^{-1}) and thigh (~1.9 %·wk^{-1}). Similarly, a 2.4 %·wk^{-1} decrease in plantar flexors ACSA was found following 5 weeks horizontal bed-rest (LeBlanc et al., 1988). Again, a 1.8 %·wk^{-1} decrease in knee extensors was observed following 8 weeks horizontal bed-rest (Mulder et al., 2006). Muscle group-specific adaptations have been demonstrated in skeletal muscle ACSA of the leg and lumbar musculature after 17 weeks of bed-rest (LeBlanc et al., 1992). The plantar-flexors were more susceptible to atrophy (~1.8 %·wk^{-1}) than the dorsiflexors (~0.9 to 1.2 %·wk^{-1}) (LeBlanc et al., 1992). The intrinsic lumbar muscles atrophied ~0.5 %·wk^{-1} but there was no significant change in psoas muscle mass (LeBlanc et al., 1992). Rittweger et al. (2005) reported a decrease in calf muscle ACSA (~2.0 %·wk^{-1}), which was greater than the reported decrease in the forearm ACSA (0.5 %·wk^{-1}) in response to 90 days bed-rest.

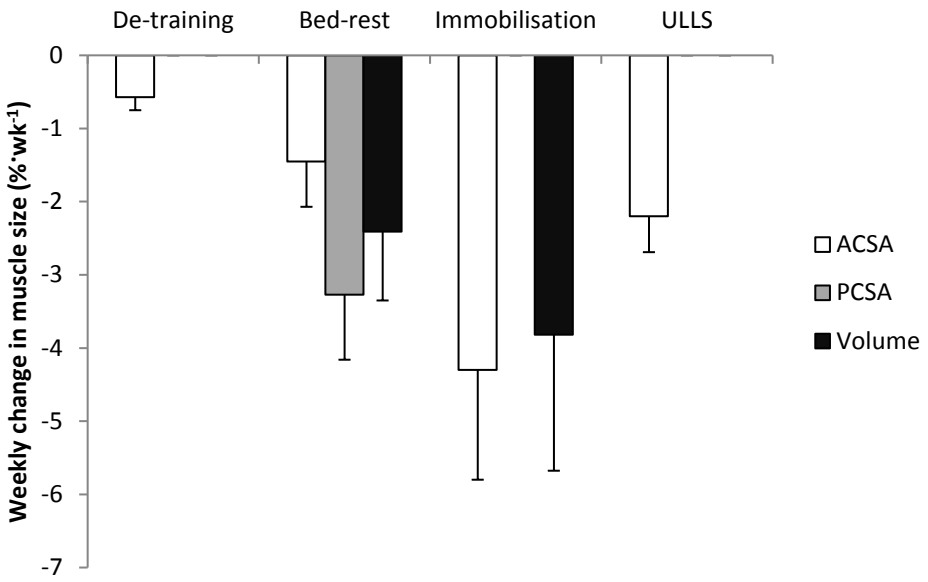


Figure 1.1. Relative change in muscle anatomical cross sectional area (ACSA), physiological cross sectional area (PCSA) and volume in response to hypo-activity models. Values are taken from the references used in the text for de-training (ACSA – 40 days and 24 weeks) (Hakkinen et al., 2000; Narici et al., 1989), bed-rest (ACSA – 30 days to 17 weeks) (Convertino, Doerr, Mathes, et al., 1989; LeBlanc et al., 1988; LeBlanc et al., 1992; Rittweger et al., 2005; Mulder et al., 2006) (PCSA – 20 days) (Akima et al., 2000; Kawakami et al., 2001; Kawakami et al., 2000; Kawashima et al., 2004) (Volume – 7 days and 32 days) (Convertino, Doerr, Mathes, et al., 1989; Ferrando et al., 1995; Krainski et al., 2014; Trappe et al., 2007), immobilisation (ACSA – 9 days to 4 weeks) (Christensen et al., 2008; Miles et al., 1994; Oates et al., 2010; Thom et al., 2001; Veldhuizen et al., 1993; White et al., 1984; Yasuda et al., 2005; Yue et al., 1997) (Volume – 2 weeks and 4 weeks) (Grosset and Onambele-Pearson, 2008; Urso, Clarkson, et al., 2006; Yue et al., 1997) and unilateral lower limb suspension (ULLS) (ACSA – 23 days and 4 weeks) (Clark et al., 2006; Clark et al., 2007; de Boer, Maganaris, et al., 2007; Seynnes et al., 2008). Where there are missing bars, this shows gaps in the literature (i.e. values are not available for a parameter during a specific hypo-activity model). Values are presented as means; error bars denote SD.

Immobilisation of the leg through plaster cast has shown to decrease calf ACSA (~3 to 5 %·wk^{-1}) (Christensen et al., 2008; White et al., 1984) and quadriceps ACSA (~5 %·wk^{-1}) (Hespel et al., 2001) after just 2 weeks. Changes in quadriceps ACSA (~8.3 %·wk^{-1}) have also been documented with as little as 10 days leg cast immobilisation (Thom et al., 2001). Similarly, Veldhuizen et al. (1993) reported decreases in quadriceps ACSA (~5.3 %·wk^{-1}) with 4 weeks leg casting. Immobilisation of the knee using a brace has also resulted in decreases in muscle ACSA (Oates et al., 2010; Yasuda et al., 2005; Dirks et al., 2014). Fourteen days of knee-brace immobilisation has resulted in ACSA decreases of the thigh (~3.1 %·wk^{-1}), quadriceps (~2.9 to 3.8 %·wk^{-1}), gastrocnemius (~4.7 %·wk^{-1}) and soleus (~3.3 %·wk^{-1}) muscles (Oates et al., 2010; Yasuda et al., 2005). Yasuda et al. (2005) found no sex-based differences in the quadriceps ACSA response to knee-brace mediated immobilisation. There is considerably less data on immobilisation-induced atrophy of the upper limb muscles. Casting of the arm for as little as 9 days has shown to decrease ACSA of the forearm (~3.2 %·wk^{-1}) (Miles et al., 1994). Yue et al. (1997) investigated the effect of 4 weeks elbow joint immobilisation with a fibre glass cast and reported a decrease in elbow flexor ACSA (~2.8 %·wk^{-1}).

Tesch et al. (1991) developed a model to study the effects of an unloaded limb in humans that allows for freely moveable joints but minimises load bearing. In this ULLS method, a sling suspends one lower leg and the contralateral shoe has an elevated sole to allow for a relaxed position of the unloaded limb. ULLS also results in decreases in muscle ACSA, though to a lesser degree than seen with immobilisation. ULLS of 23 days has been reported to decrease knee extensor (~3 %·wk^{-1}) (de Boer, Maganaris, et al., 2007) and plantar flexor (~2.7 %·wk^{-1}) (Seynnes et al., 2008) ACSA. Correspondingly, decreases in plantar flexor (~2.0 to 2.3 %·wk^{-1} and 1.6 %·wk^{-1}) and knee extensor (~2.0 %·wk^{-1} and 1.9 %·wk^{-}

[1]) ACSA have been reported in response to 4 weeks and 30 days of ULLS, respectively (Clark et al., 2006; Clark et al., 2007; Cook et al., 2014). It would therefore, seem that in terms of ACSA at least, the most impactful model of hypo-activity is immobilisation.

1.1.2. Muscle physiological cross sectional area

Physiological cross-sectional area (PCSA) is the area of the muscle at right angles to the longitudinal axis of the fibres. Muscle PCSA has been associated with the maximal force generating capacity of a muscle (Powell et al., 1984) and has been shown to decrease with bed-rest (Akima et al., 2000; Kawakami et al., 2001; Kawakami et al., 2000). Twenty days bed-rest has been shown to decrease PCSA of the thigh (between ~2.7 to 3.6 %·wk^{-1}) and adductor muscle group (~2.3 %·wk^{-1}) (Kawakami et al., 2001; Kawakami et al., 2000; Kawashima et al., 2004). Akima et al. (2000) demonstrated muscle group-specific adaptations, demonstrating a decrease in PCSA of knee extensor (~2.5 %·wk^{-1}), knee flexor (~4.0 %·wk^{-1}) and plantar flexor (~4.5 %·wk^{-1}) muscles in response to 20 days of 6 degrees head-down-tilt bed-rest. It is generally accepted that muscle losses are greater in the knee extensors than the knee flexors after unloading in humans (Bloomfield, 1997). Akima et al. (2000) demonstrated the opposite to this, which could be due to the methodology used to determine PCSA. In addition, since a muscle placed in a shortened position experiences a greater degree of atrophy than one placed in a lengthened position (Williams and Goldspink, 1973), the pattern/magnitude of disuse would, therefore, be expected to be modulated by both the mode of hypo-activity and the joint angle adopted in the immobilisation. Bed-rest, however, had no effect on the PCSA of the tibialis anterior (Akima et al., 2000). The tibialis anterior experiences lower activation during habitual physical activities than other muscles such as the plantar flexor, and as such may explain the lack of decrease

in tibialis anterior muscle PCSA with bed-rest. Comparisons of bed-rest to other hypo-activity models in terms of PCSA changes is not yet possible, as research is lacking with this parameter being measured.

1.1.3. Muscle volume

Muscle volume is a major determinant of joint torque (Fukunaga et al., 2001) and has been shown to decrease in response to bed-rest and immobilisation models (Convertino, Doerr, Mathes, et al., 1989; Ferrando et al., 1995; Grosset and Onambele-Pearson, 2008; Urso, Clarkson, et al., 2006; Yue et al., 1997). Muscle volume of the thigh decreases (~3 %·wk^{-1}) with as little as 7 days bed-rest (Ferrando et al., 1995). Following 30 days bed-rest Convertino et al. (1989) reported decreases in calculated leg volumes of the calf (~2.3 %·wk^{-1}) and thigh (~1.1 %·wk^{-1}). Similarly, significant decreases in quadriceps (-1.8 %·wk^{-1} and -2.45 %·wk^{-1}) and calf (-3.8 %·wk^{-1}) muscle volume have been reported with 5 and 8 weeks of head-down-tilt bed-rest, respectively (Krainski et al., 2014; Trappe et al., 2007). Yue et al. (1997) investigated the effect of 4 weeks elbow joint immobilisation with a fibre glass cast and reported a decrease in elbow flexor volume (~2.9 %·wk^{-1}). A case study of a fracture patient who fractured the fifth metatarsal of the right foot displayed substantial and rapid losses in muscle volumes, both proximally and distally to the immobilisation site after 4 weeks of subsequent immobilisation (Grosset and Onambele-Pearson, 2008). The degree of muscle volume decrease varied between the different muscle sites of the triceps surae (~5.5 %·wk^{-1}), quadriceps (~6.0 %·wk^{-1}) and hamstrings (~1.6 %·wk^{-1}) (Grosset and Onambele-Pearson, 2008). This is in agreement with the general acceptance that muscle volume is lost to a greater extent in the knee extensors compared to the knee flexors (Bloomfield, 1997). Urso et al. (2006) demonstrated different responses to 2 weeks adductor pollicis immobilisation between younger

and older males. Adductor pollicis volume decreased ~2.1 %·wk^{-1} (not significant) in young males and significantly decreased by ~4.8 %·wk^{-1} in older males (Urso, Clarkson, et al., 2006).

1.1.4. Upper vs. lower limb

Immobilisation through casting appears to have a greater effect on the lower limb musculature than the upper body. This is not surprising since the habitual loading of the lower extremities, because of body weight in normal ambulation and even in the absence of intended physical exertion, is far more substantial than that in the upper extremities. Understandably, this thereby affects the required threshold of decrease in muscle activity necessary to negatively impact on muscle metabolism. (Table 1.1). Forearm muscle ACSA decreased 4.1 % with 9 days arm casting (Miles et al., 1994), whereas, a similar period of immobilisation of the lower limb with 10 days casting resulted in an 11.8 % decrease in quadriceps ACSA (Thom et al., 2001). Similarly, with longer periods of immobilisation the effect seems to be greater in the lower limbs. In response to 4 weeks elbow joint casting, Yue et al. (1997) reported an 11.2 % decrease in elbow flexor ACSA, whereas, Veldhuizen et al. (1993) reported a 21 % decrease in quadriceps ACSA in response to 4 weeks leg casting.

Table 1.1. Relative change in muscle anatomical cross sectional area (ACSA), physiological cross sectional area (PCSA) and volume in response to hypo-activity models. Values are separated into the effect of each hypo-activity model on the upper limb (UL) versus the lower limb (LL). The values are taken from the references used in the text for de-training, bed-rest, immobilisation and ULLS. Where there are missing values, this shows gaps in the literature (i.e. values are not available for a parameter during a specific hypo-activity model).

	ACSA_UL (%/week)	ACSA_LL (%/week)	PCSA_UL (%/week)	PCSA_LL (%/week)	Volume_UL (%/week)	Volume_LL (%/week)
De-training	-	-0.6	-	-	-	-
Bed-rest	-0.5	-1.6	-	-3.3	-	-2.4
Immobilisation	-3.0	-4.5	-	-	-3.3	-4.4
ULLS	-	-2.4	-	-	-	-
Mean (SD) of 4 models	-1.8 (1.8)	-2.3 (1.7)		-3.3 (0.01)	-3.3 (0.01)	-3.4 (1.4)

1.1.5. Intermuscular adipose tissue

Using signal intensity analysis of lower limb magnetic resonance images (MRI), Manini et al. (2007) discriminated between the relative changes in adipose and skeletal muscle tissue resulting from a 4-week period of ULLS. In addition to the characteristic reduction in muscle ACSA, there was a concomitant 15 % increase in intermuscular adipose content after 4 weeks of lower limb suspension (Manini et al., 2007). Thus, these findings suggest that hypo-activity induced alterations in skeletal muscle morphology goes beyond muscle atrophy alone.

1.1.6. Summary: muscle morphology

Together, these findings show that the extent of muscle atrophy differs depending on the hypo-activity model. Certain factors may modulate the differential responses to hypo-activity models (e.g. age, nutritional status). Indeed, both Kortebein et al. (2007) and Yue et al. (2006) suggested that older individuals experience greater losses in muscle mass when compared to younger individuals. A change in nutritional status, whether it is due to physiological changes directly caused by hypo-activity or to altered behaviour that is caused by hypo-activity and leads to changes in diet, could affect the physiological systems in question. The above also suggest that the degree of muscle atrophy differs between muscle groups, with the leg and postural muscles being most susceptible to atrophy. This is likely to be due to the comparatively substantial decrease in habitual weight-bearing forces applied to the lower limb during hypo-activity. Hypo-activity not only decreases muscle content, but also impacts on the intrinsic composition of the said skeletal muscle through increased adiposity (Manini et al., 2007) and altered muscle architecture (de Boer et al., 2008). Fibre type differences have been

reported in response to hypo-activity, with a transition from slow to fast fibre types (Trappe et al., 2004).

The decrease in muscle mass seen with hypo-activity may be the result of an imbalance between protein synthesis and protein breakdown (Ferrando et al., 1996; Gibson et al., 1987; Urso, Scrimgeour, et al., 2006). In response to 14 days simulated microgravity, Ferrando et al. (1996) reported a loss of lean muscle mass, accompanied with a 14 % decrease in protein synthesis and no change in protein breakdown. Similarly, Gibson et al. (1987) reported a marked fall in muscle protein synthesis in response to 7 weeks leg immobilisation. A shorter period of immobilisation (21 days) provided little evidence of increases in messenger ribonucleic acid (mRNA) for catabolic enzymes or increases in enzyme activity during this period (de Boer, Selby, et al., 2007). However, there is some evidence to suggest that increases in catabolic potential do occur, and that this event happens very quickly (48 hours) after immobilisation (Urso, Scrimgeour, et al., 2006). Nevertheless, collectively the evidence suggests that protein breakdown is unlikely to be a key modulator in the process of muscle atrophy occurring during immobilisation in humans (Rennie, 2009; Rennie et al., 2010).

The molecular signalling responses to de-training are only just beginning to be investigated, and to date, only changes in metabolic proteins have been reported in human skeletal muscle (Burgomaster et al., 2007; Simsolo et al., 1993). With bed-rest, Ogawa et al. (2006) reported increased mRNA expression of the E3 ligases, Cbl-b and Atrogin-1 in response to 20 days bed-rest. This was accompanied by a threefold increase in ubiquitinated proteins (Ogawa et al., 2006). Investigation into the effects of limb immobilisation on cell signalling in humans is limited. Modest changes in mRNA for many genes in the first 2 days after immobilisation have been reported but these changes do not affect protein levels of most transcripts (Urso, Scrimgeour, et al., 2006). However, the Akt

protein synthesis pathway and extracellular matrix components seem to be affected within 48 hours of immobilisation (Urso, Scrimgeour, et al., 2006). Chen et al. (2007) and Jones et al. (2004) reported increases in the E3 ligases, Atrogin-1 and muscle ring finger 1 (MuRF-1) in response to 11 to 14 days immobilisation in humans. These changes were not seen with 48 hours of immobilisation (Urso, Scrimgeour, et al., 2006) and are, therefore, thought to only occur after long duration (days rather than hours) immobilisation. Increased metallothionein expression in human skeletal muscle fibres has been associated with exposure to physiological stress, which results in elevated levels of reactive oxygen species (ROS) (Penkowa et al., 2005). Urso et al. (2006) reported a more than two-fold increase in metallothioneins in human skeletal muscle with 48 hours of immobilisation. However, neither Chen et al. (2007) nor Jones et al. (2004) identified changes with longer periods of immobilisation. This may suggest that metallothioneins are increased in the first few days of hypo-activity to prevent ROS-mediated DNA or cellular damage. De Boer et al. (2007) investigated the effects of ULLS on gene expression and cell signalling. They reported increased expression of mRNA for MuRF-1 by approximately threefold after 10 days without changes in MAFbx or tripeptidyl peptidase II mRNA, but all decreased between 10 and 21 days (de Boer, Selby, et al., 2007). These authors concluded that both myofibrillar and tendon protein synthetic rates show progressive decreases during 21 days of disuse; in muscle this is accompanied by decreased phosphorylation of focal adhesion kinase (FAK), with no marked increases in genes for proteolytic enzymes (de Boer, Selby, et al., 2007). Overall, whilst it is clear that cell signalling responses differ between hypo-activity models; further research is needed to provide a definitive description of the timing, magnitude and nature of these molecular adaptations.

1.2. Muscle strength

The associated decline in strength through hypo-activity can be best described based on the mode of assessment. Both isometric and dynamic strength have been reported to decline with hypo-activity, the relative magnitude of which appears to largely reflect the patterns of atrophy described above.

1.2.1. Isometric strength

Hypo-activity models alter muscular isometric torque. After 40 days de-training, Narici et al. (1989) reported a decrease in knee extension isometric MVC (~2.1 %·wk^{-1}). Similarly, maximal isometric quadriceps strength has been reported to decrease with 90 days de-training (~1.3 %·wk^{-1}) (Andersen and Aagaard, 2000). More dramatic losses in isometric torque are seen with stricter hypo-activity models. Bed-rest models have been shown to decrease maximal voluntary force of plantar flexion (~2.4 to 7.5 %·wk^{-1}) (Kamiya et al., 2004; Blottner et al., 2014; Krainski et al., 2014) and knee extensor torque (~2.1 to 5.0 %·wk^{-1}) (Berg et al., 1997; Krainski et al., 2014; Mulder et al., 2006). Correspondingly, Kawakami et al. (2001) showed a decrease in muscle force for knee extension (~3.8 %·wk^{-1}) with 20 days bed-rest.

Studies using 2 weeks of cast immobilisation have reported decreases in triceps surae isometric MVC torque (~8.5 and 12 %·wk^{-1}) (Gondin et al., 2004; White et al., 1984). A discrepancy between the magnitudes of change in the two studies may be due to the degree of immobilisation. Gondin et al. (2004) simply immobilised the ankle joint, whilst, White et al. (1984) utilised a full leg cast. In the full leg cast a larger muscle mass is immobilised as compared to that of the ankle joint immobilisation, therefore, resulting in greater relative decreases in MVC torque. A significant decrease in quadriceps isometric MVC strength has been

reported with as little as 5 days knee immobilisation (Dirks et al., 2014). Knee-brace mediated immobilisation has resulted in a decrease in knee extensor and plantar flexion isometric strength (~11.2 and 12.7 %·wk^{-1}, respectively) (Oates et al., 2010). Knee-cast mediated immobilisation resulted in a slightly larger decrease in isometric leg strength (~15.7 %·wk^{-1}) (Hortobagyi et al., 2000). Christensen et al. (2008) utilised a knee-to-toe plaster cast and reported a decrease in isometric calf muscle strength (~4.5 %·wk^{-1}). Studies using casting to immobilise the elbow joint have found decreases in isometric MVC of the elbow flexors (~5.3 to 8.8 %·wk^{-1}) (Semmler et al., 2000; Vaughan, 1989; Yue et al., 1997), and a decrease in the maximum load that could be lifted (Yue et al., 1997). A more dramatic decrease in isometric MVC torque has been reported in the flexors and extensors of the wrist (~22.8 to 25.3 %·wk^{-1}) in response to immobilisation (Lundbye-Jensen and Nielsen, 2008; Miles et al., 1994).

With ULLS, isometric torque appears to be affected to a lesser degree than with immobilisation models. An explanation for the above observation may be that ULLS removes weight bearing but allows for freely moveable joints (hence a degree of muscular activity) whereas immobilisation is a more rigid model that does not allow joint movement (hence a greater restriction of muscular activity). Studies have reported plantar flexor (~5 to 7 %·wk^{-1}) (Clark et al., 2006; Seynnes et al., 2008; Cook et al., 2014) and knee extensor (3.7 %·wk^{-1}) (Cook et al., 2014) isometric MVC torque to decrease with ULLS. With ULLS, increased fluctuations in plantar flexion (~3 %·wk^{-1}) and knee extension (~5.5 %·wk^{-1}) isometric force have been demonstrated (Clark et al., 2007).

1.2.2. Isokinetic strength

In addition to the established decline in isometric strength (torque and force), hypo-activity models also result in reductions in dynamic torque outputs. After 14

days de-training isokinetic eccentric and concentric knee extension force has been shown to decrease by ~6 and 1.2 %·wk^{-1}, respectively (Hortobagyi et al., 1993). With as little as 14 days bed-rest decrements in knee extensor 1 repetition maximum (~4.5 %·wk^{-1}) are seen along with a fall in MVC (~7.5 %·wk^{-1}) (Bamman et al., 1998). After 6 weeks bed-rest, maximal voluntary concentric knee extensor torque was shown to decrease uniformly across angular velocities (~4.1 to 5.0 %·wk^{-1}) (Berg et al., 1997). Muscle-specific adaptations are evident with bed-rest, as shown by Dudley et al. (1989) who reported a decrease in concentric and eccentric isokinetic knee extensor peak torque (~4.4 %·wk^{-1}), with no alterations in knee flexors in response to 30 days 6 degrees head-down-tilt bed-rest. Again, muscle-specific adaptations were demonstrated by Le Blanc et al. who reported a decrease in plantar flexor concentric isokinetic strength (~2.6 %·wk^{-1}) and no change in the isokinetic strength of the dorsiflexors with 5 weeks bed-rest (LeBlanc et al., 1988). As with the knee extensors vs. knee flexors difference in sensitivity to hypo-activity alluded to above, the plantar flexor muscles experience a greater level of recruitment during gait than the tibialis anterior. Thus, habitual muscle recruitment prior to hypo-activity would appear to be a large determinant of the relative magnitude of hypo-activity-induced changes.

Results from lower limb immobilisation models indicate that short-term immobilisation is associated not only with atrophy, but also with a diminished capacity of the muscle to perform both concentric and eccentric strength (Hortobagyi et al., 2000; Miles et al., 1994). Lower limb casting results in a dramatic decrease in isokinetic quadriceps strength (~12.5 to 29.1 %·wk^{-1}) (Thom et al., 2001; Hespel et al., 2001) and isokinetic knee extensor torque (~15.5 %·wk^{-1}) (Kubota et al., 2011). There is evidence that the effect of leg cast immobilisation on isokinetic strength of the knee extensors and flexors is greater in the knee extensors, demonstrated by a fall in peak torque of ~13.3 %·wk^{-1} for the knee

extensors and ~3.3 %·wk^{-1} for knee flexors (Veldhuizen et al., 1993). Cast immobilisation of the arm also results in decreased concentric (~6.9 to 16.9 %·wk^{-1}) and eccentric (~9.7 to 14.4 %·wk^{-1}) strength for flexion, extension, pronation and supination of the wrist (Miles et al., 1994).

Less dramatic decreases in isokinetic strength are seen with ULLS compared to immobilisation. De Boer et al. (2007) found a decrease in isokinetic knee extensor torque in response to 23 days ULLS (~6.4 %·wk^{-1}). Similarly, after 4 weeks ULLS mean average peak isokinetic torque is decreased (~4.3 %·wk^{-1}) (Berg, Dudley, et al., 1993). With as little as 14 days ULLS, a decrease in peak isokinetic torque (~5 to 8.6 %·wk^{-1}) and total work performed (~7.5 to 10.0 %·wk^{-1}) by knee extensors and flexors was reported (Deschenes et al., 2002).

1.2.3. Strength vs. size changes

There is evidence to suggest that decreases seen in strength in response to hypo-activity models are greater than the changes seen in muscle size. With de-training the loss in leg muscle ACSA (~0.7 %·wk^{-1}) was not as great as the decrease seen in knee extension MVC (~2.1 %·wk^{-1}) (Narici et al., 1989). Similarly, in bed-rest Kawakami et al. (2001) suggested that the decrease in knee extension mean muscle force (~3.8 %·wk^{-1}) seen after 20 days head-down-tilt bed-rest was related more to changes in neural activation than those in PCSA (~2.7 %·wk^{-1}). Correspondingly, Berg et al. (1997) suggested that the decline seen in strength (~4.1 to 5.0 %·wk^{-1}) could not be entirely accounted for by decreased ACSA (~2.3 %·wk^{-1}), and that the strength loss could also be due to factors resulting in decreased neural input to muscle and/or reduced specific tension of muscle, as evidenced by a decreased torque to EMG ratio. Discrepancies between decreases in muscle size and muscle strength have also been reported in upper and lower limb immobilisation studies. White et al. (1984) reported a ~5 %·wk^{-1} decrease in

muscle ACSA whilst triceps surae MVC decreased ~12 %·wk^{-1}. Additionally, the upper limb decreases in forearm ACSA (~3.2 %·wk^{-1}) were much smaller than those reported in forearm flexor and extensor strength (~22.8 to 25.3 %·wk^{-1}) (Miles et al., 1994). Again, in ULLS models muscle torque (~5 to 7 %·wk^{-1}) appears to decrease to a greater degree than muscle ACSA (~2.3 to 2.7 %·wk^{-1}) (Clark et al., 2006; Seynnes et al., 2008).

1.2.4. Summary: muscle strength

Bed-rest appears to have varying degrees of impact on the upper and lower body. After 14 days of 6 degrees head-down-tilt bed-rest maximum voluntary force for plantar flexion was decreased (~7.5 %·wk^{-1}) whilst no effect was observed on maximal voluntary force of handgrip (Kamiya et al., 2004). Similar results were demonstrated by LeBlanc et al. (1992) who showed after 17 weeks of continuous bed-rest that isokinetic muscle strength decreased significantly in the thigh and calf with no loss in the arms. These results further support the idea that the lower limbs are primarily affected by bed-rest, more so than the upper limb. However, Gogia et al. (1988) did observe a decrease in elbow flexor torque (~3.8 %·wk^{-1}) and a non-significant decrease in elbow extension torque (~1.4 %·wk^{-1}) after 5 weeks of bed-rest. Thus, suggesting that strength in the upper limb is affected by bed-rest but only in specific muscles, during specific tasks.

Together, these findings show that in addition to the reduction in muscle mass, hypo-activity also results in a dramatic loss of strength (Figure 1.2). Models in which the joint is immobilised appear to have a greater impact on strength than unloading models. These changes in muscular strength vary between hypo-activity models. The degree of loss in muscular strength surpasses the loss of muscle mass. Therefore, other alterations in the neuromuscular system, other than the reduction in contractile proteins must contribute to the excessive loss of strength.

Voluntary force production is associated with neurological (e.g. EMG activity) and skeletal muscle (fibre type, connective tissue, oxygen supply to skeletal muscle) properties, thus suggesting these two factors as mechanisms accounting for the loss of strength with hypo-activity models.

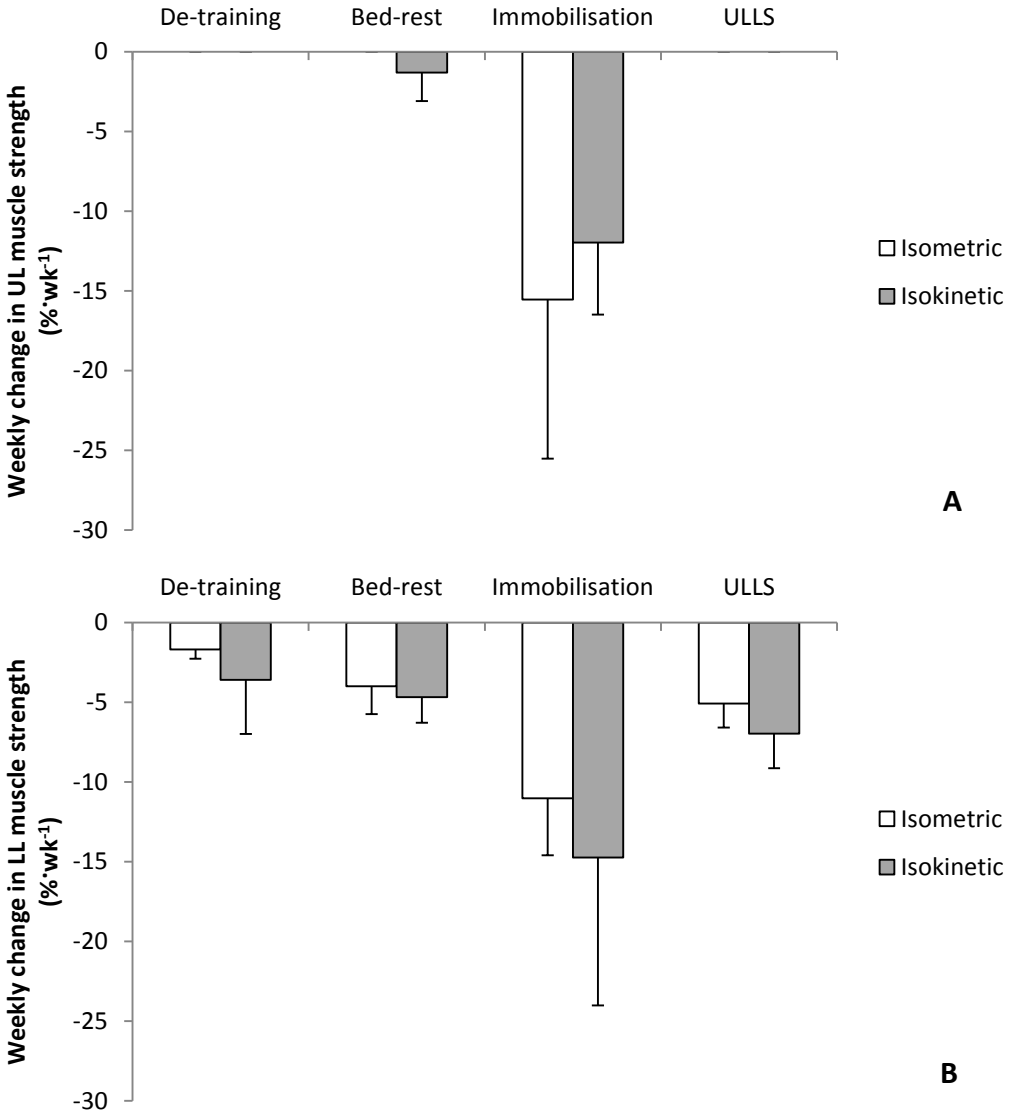


Figure 1.2. Relative change in isometric and isokinetic strength in response to hypo-activity models. (A) Values taken from references in the text for upper limb (UL) changes in strength in response to de-training, bed-rest (isokinetic) (Gogia et al., 1988; Kamiya et al., 2004; LeBlanc et al., 1992), immobilisation (isometric) (Lundbye-Jensen and Nielsen, 2008; Miles et al., 1994; Semmler et al., 2000;

Vaughan, 1989; Yue et al., 1997) (isokinetic) (Miles et al., 1994) and ULLS. (B) Values taken from references in the text for lower limb (LL) changes in strength in response to de-training (isometric) (Andersen and Aagaard, 2000; Narici et al., 1989) (isokinetic) (Hortobagyi et al., 1993), bed-rest (isometric) (Berg et al., 1997; Kamiya et al., 2004; Kawakami et al., 2001; Blottner et al., 2006; Krainski et al., 2014; Mulder et al., 2006) (isokinetic) (Bamman et al., 1998; Berg et al., 1997; Dudley et al., 1989; LeBlanc et al., 1988), immobilisation (isometric) (Christensen et al., 2008; Gondin et al., 2004; Hortobagyi et al., 2000; Oates et al., 2010; White et al., 1984; Dirks et al., 2014; Hespel et al., 2001; Kubota et al., 2011) (isokinetic) (Thom et al., 2001; Veldhuizen et al., 1993) and ULLS (isometric) (Clark et al., 2006; Clark et al., 2007; Seynnes et al., 2008; Cook et al., 2014) (isokinetic) (Berg, Dudley, et al., 1993; de Boer, Maganaris, et al., 2007; Deschenes et al., 2002). Where there are missing bars, this shows gaps in the literature (i.e. values are not available for that parameter during a specific hypo-activity models). Values are presented as means; error bars denote SD.

1.3. Muscle fatigability

Studies have also examined the impact of hypo-activity models on the fatigability of skeletal muscle. Kamiya et al. (2000) showed no change in time to fatigue after 14 days bed-rest. After a longer period of bed-rest (8 weeks), Mulder et al. (2007) demonstrated an increase in fatigability (7.2-10.2 %/min decrease in maximum voluntary isometric torque per minute exercise; or ~0.9-1.3 $\%\cdot wk^{-1}$ fatigability increment). The contrast between the two studies would tend to suggest a delay in the impact of hypo-activity on muscle fatigability.

The effect of immobilising a limb has various different effects on skeletal muscle fatigability. Two weeks of full leg cast immobilisation resulted in no effect on muscle fatigability (White et al., 1984). In contrast, Veldhuizen et al. (1993) found a decrease in isokinetic quadriceps endurance work from 9.1 kJ to 5.6 kJ after 4 weeks leg cast immobilisation. These results suggest that short periods of lower limb immobilisation (≤2 weeks) have little effect on muscle fatigability whilst longer periods of immobilisation (≥4 weeks) increases muscle fatigability. Studies investigating the effects of immobilisation on skeletal muscle fatigability in the upper limbs have found different effects to those in the lower limbs. Similar to lower limbs shorter periods of immobilisation in the upper limbs appear to have minimal effects on muscle fatigability (Miles et al., 1994). Unlike the lower limb, longer periods of immobilisation of the upper limb show a trend towards increased resistance to fatigability. Following 3 weeks of hand-forearm immobilisation time to task failure increased by 21 % (~7 $\%\cdot wk^{-1}$) (Clark et al., 2008). Semmler et al. (2000) investigated the effects of fiberglass cast immobilisation of the elbow joint, and reported 7 out of the 12 immobilised participants exhibited an unusual pattern of muscle activity during a fatiguing contraction after immobilisation. In those individuals with this unusual pattern of muscle activity there was an associated increase in the ability to maintain a contraction over an extended period of time in the elbow flexor muscles (Semmler et al., 2000). The physiological basis for the sometimes-observed immobilisation-induced decreased fatigability is not clear but it is likely to be related to neural factors (Semmler et al., 2000). In contrast to this, Miles et al. (2005) found an increase in fatigability in response to 3 weeks arm suspension in untrained but not trained individuals. Previous research showed that ULLS led to increased fatigability after 4 weeks of unloading (Berg, Dudley, et al., 1993). Results from Deschenes et al. (2002) found a contrasting decrease in fatigability after just 2 weeks of unloading.

Collectively these results suggest that muscle fatigability varies between different hypo-activity models (Figure 1.3). Shorter periods of hypo-activity (≤2 weeks) generally appear to have little impact on fatigability. Muscle fatigability appears to increase in weight-bearing muscles but immobilisation in the upper limbs suggests an increase in resistance to fatigue. Differences between studies could be due to the duration of unloading or in the method used to test fatigue resistance. The mechanisms that cause fatigue are specific to the task being performed (Enoka and Duchateau, 2008; Hunter et al., 2004). Therefore, variability between fatigue resistance responses to hypo-activity models may be due to task specificity. Studies investigating a comparison of different fatigue tasks before and after hypo-activity are sparse. Yue et al. (1997) demonstrated a task-dependent effect on muscle fatigue with substantially increased endurance time (reduced fatigability) at a low force (20 % MVC) and no statistical effect at a moderate force (65 % MVC) in the elbow flexors. The selective improvement of fatigue resistance for the low-force contraction was accompanied by the absence of a change in the time course of the twitch, suggesting that the immobilisation-induced adaptation included an improved efficacy of some excitation-contraction processes and underscored the major role of these mechanisms in determining the endurance time for low-force, long-duration contractions. It appears that the hypo-activity induced adaptations in muscle fatigability vary with the specifics of the task being performed. More research is needed to investigate these task-specific responses to different models of hypo-activity.

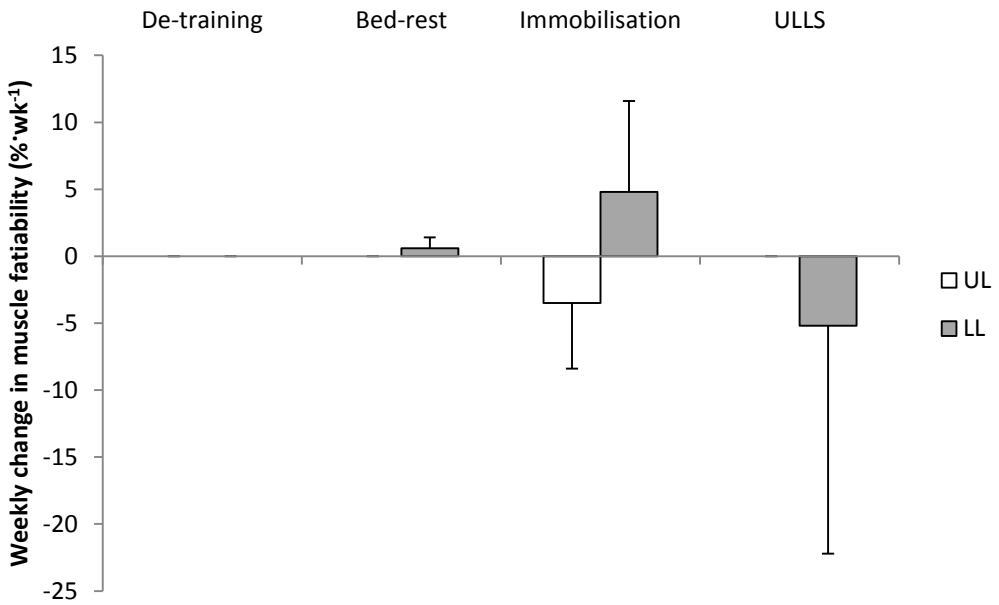


Figure 1.3. Relative change in muscle fatigability in response to hypo-activity models (mean ± SD). Positive percentage change depicts an increase in fatigability whilst negative percentage change shows a decrease in fatigability. Values are separated into the effect of each hypo-activity model on the upper limb (UL) versus the lower limb (LL). The values are taken from the references used in the text for bed-rest (LL) (Kamiya et al., 2000; Mulder et al., 2007), immobilisation (UL) (Clark et al., 2008; Miles et al., 1994) (LL) (Veldhuizen et al., 1993; White et al., 1984) and ULLS (LL) (Berg, Dudley, et al., 1993; Deschenes et al., 2002). Where there are missing bars, this shows gaps in the literature (i.e. values are not available for a parameter during a specific hypo-activity model).

Numerous adaptations in fatigue mechanisms have been hypothesised to explain the observed preservation and decrease in fatigability in response to hypo-activity. As stated previously, hypo-activity results in muscle atrophy and a decrease in muscle strength, has been reported to be accompanied by myofiber transitions from slow to fast (Fitts et al., 2000) and a shift in fuel metabolism away from lipid fuels toward glycolysis (Stein and Wade, 2005). Typically, these changes are associated with increased fatigability. Cardiovascular adaptations with hypo-activity (Convertino, 1997) reduces oxygen delivery and oxygen utilization which may impair prolonged exercise capacity. Additionally, exercise tolerance may be influenced by impaired muscle activation after hypo-activity (Gondin et al., 2004; Kawakami et al., 2001). In light of this, the reports of decreased fatigability with hypo-activity are puzzling, and the underlying mechanisms remain unclear. It is possible that an atrophy-induced decrease in absolute force production will result in decreased intramuscular pressure. This in turn, will increase blood flow to the muscle and increase supply to match the metabolic demand (Bodor, 2002; Semmler et al., 2000). Other potential mechanisms include adaptations in the neural activation strategy utilised (Semmler et al., 2000), adaptations in the basal inorganic phosphate concentration (Shaffer et al., 2000), and changes in excitation-contraction coupling (Yue et al., 1997).

1.4. Nutritional supplementation

As mentioned above, there is strong evidence that protein synthesis is decreased in response to periods of bed-rest and immobilisation (de Boer, Selby, et al., 2007; Ferrando et al., 1996; Gibson et al., 1987). That resistance exercise provides an anabolic stimulus during hypo-activity is undisputed (Akima et al., 2000; Bamman et al., 1998; Ferrando et al., 1997). When supplemented with nutritional

interventions, the benefits of exercise during bed-rest appear additive (Brooks et al., 2008), thereby suggesting synergistic pathways for counteracting atrophy. It may not always be practical to prescribe exercise to counteract the atrophy brought about by inactivity. In these cases, such as trauma, pharmaceuticals may be used and have been tried with varying degrees of success (Jones et al., 2009). However, effective long-term medication is not a palatable option (e.g. costs, side effects, repeated injections). Where exercise is not a practical prescription, supplementing the diet with potential/recognised hypertrophic nutrients may be an effective and easily adhered to intervention programme for preventing the loss of muscle mass/function seen with hypo-activity. In this latter therapeutic group, potential candidates include proteins (essential amino acids (EAA) and Leucine in particular), creatine, omega-3 (ω-3) fatty acids, vitamin D and antioxidants, to name but a few (Kim et al., 2010; Sakuma and Yamaguchi, 2012).

1.4.1. Protein

Stuart et al. (1990) sought to determine whether the catabolic effects of bed-rest in humans was due to a decrease in protein synthesis, and if so, to assess whether increasing the amount of dietary protein might be beneficial. The calculated non-oxidative Leucine disappearance was used as a measure of whole-body-protein synthesis, which was shown to decrease when dietary protein was low. Bed-rest resulted in a 24 % decrease in nonoxidative Leucine disappearance in participants assigned to a lower-protein diet (0.6 g protein·kg body $wt^{-1}·d^{-1}$), whereas Leucine kinetics were unchanged by the same bed-rest protocol in participants who received a higher-protein diet (1.0 g protein·kg body $wt^{-1}·d^{-1}$) (Stuart et al., 1990). In other words, whereas protein synthesis is suggested here to decrease with bed-rest, dietary supplementation of protein appears to protect against this deleterious response. Even short periods of muscle disuse have been shown an early

catabolic molecular signalling response accompanying a substantial loss of skeletal muscle mass and strength (Wall et al., 2014). More recently, Wall et al. (2013) reported that knee immobilisation impaired the muscle protein synthetic response to dietary protein intake in vivo in healthy young men.

1.4.2. Essential amino acids

The ingestion of a mixed meal can switch human muscle protein balance from negative to positive (Rennie et al., 1982). This stimulation of muscle protein synthesis with a mixed meal appears to be mainly attributable to the increased availability of amino acids; therefore, amino acids alone appear to be able to stimulate muscle protein synthesis (Bennet et al., 1989; Fryburg et al., 1995; Smith et al., 1992). Studies have demonstrated directly that whey proteins and their constituent amino acids efficiently promote protein synthesis (Bos et al., 1999; Fouillet et al., 2002). The ingestion of only EAAs is sufficient for the acute stimulation of muscle protein synthesis (Børsheim et al., 2002). Bolus oral ingestion of EAAs produces a several-fold increase in plasma amino acid levels (Paddon-Jones, Sheffield-Moore, Zhang, et al., 2004) and has been shown to stimulate net protein synthesis to a greater extent than a mixed meal or a solution containing nonessential amino acids (Tipton et al., 1999). Studies have shown that providing a nutritional supplement enriched with EAAs could improve lean body mass, strength and physical function even without exercise (Katsanos et al., 2006).

Previous studies have shown improved nitrogen balance during both 6 and 14 days of bed-rest when provided with a daily supplementation of 11 g of branch-chain amino acids (BCAA), compared with the same dose of nonessential amino acids (Stein et al., 2003; Stein et al., 1999). It appears that a greater dose of EAAs (49.5 $g \cdot d^{-1}$) during 28 days bed-rest prevented any noticeable changes in muscle

mass (Paddon-Jones, Sheffield-Moore, Urban, et al., 2004). Paddon-Jones et al. (2004) however, reported that during this 28-day period, that although no changes in muscle mass were observed they did find a decline in muscle strength. Nonetheless, the decrease in muscle strength with EAAs (11 %) was still noticeably less than the decrease in strength seen in the control group (23 %) (Paddon-Jones, Sheffield-Moore, Urban, et al., 2004). These results collectively demonstrate a positive effect of EAAs supplementation during periods of bed-rest ranging from 6 to 28 days on both muscle mass and function (Paddon-Jones, Sheffield-Moore, Urban, et al., 2004; Stein et al., 2003; Stein et al., 1999).

1.4.3. Creatine

Creatine supplementation is another potential supplement that may attenuate hypo-activity induced decreases in muscle size and strength. Johnston et al. (2009) reported that short-term (29 days) creatine supplementation (20 $g \cdot d^{-1}$) attenuates the loss in muscle mass and strength during upper arm immobilisation. It is well known that muscle total creatine content can be rapidly raised by a high-dose oral creatine intake (Harris et al., 1992) and that long-term creatine intake can enhance the effects of weight training on muscle size and strength (Terjung et al., 2000; Vandenberghe et al., 1997). Creatine supplementation during 10 weeks of resistance training has been shown to accelerate the rate of muscle hypertrophy in young adults who previously had their knee flexors immobilised for 2 weeks (Hespel et al., 2001). Furthermore, 14 days creatine supplementation during hind-limb immobilisation lessened the rate of loss in the plantarflexors in a rodent model (Aoki et al., 2004). Additionally, Op't Eijinde et al. (2001) showed that creatine supplementation prevented the loss of glucose transporter type 4 (GLUT4) during muscle disuse and increased muscle GLUT4 content above normal levels during subsequent rehabilitation. Collectively these studies suggest that creatine

supplementation during resistance training and rest may be effective at reversing or maintaining lower-body muscle mass during and after an immobilised state.

1.4.4. Antioxidants

Intricate antioxidant defence systems in the body work to continually manage oxidative stress. To counteract ROS, enzymatic and nonenzymatic antioxidants work together (Mates et al., 1999). Enzymes work to improve or maintain an antioxidant balance and to avert oxidative damage by scavenging or preventing transformation of ROS to intracellular molecules and inhibiting their conversion to more deleterious forms. Endogenous nonenzymatic antioxidants such as vitamins C and E, carotenoids and flavonoids play important roles by contributing to the antioxidant system as cofactors for antioxidant enzymes. Results from Zwart et al. (2009) provide evidence that increased oxidative stress occurs during bed-rest. These data are also supported by results of several other studies that show evidence for elevated oxidative stress and increased ROS (Pawlak, Kedziora, Zolynski, Kedziora-Kornatowska, Blaszczyk andWitkowski, 1998; Pawlak, Kedziora, Zolynski, Kedziora-Kornatowska, Blaszczyk, Witkowski, et al., 1998; Zezerov et al., 1989). It would be interesting to see whether antioxidant supplementation during hypo-activity models will have beneficial effects on these outcome measures and furthermore, see whether this would then result in the attenuation of muscle loss in these models.

1.4.5. Vitamin D

Ceglia (2009) proposed vitamin D supplementation as an effective nutritional intervention to attenuate age related sarcopenia. Vitamin D is required to absorb calcium and phosphorus in the body and serves several important functions. It plays a crucial role in maintaining bone, muscle function, modulation of cell

growth, neuromuscular and immune function. Vitamin D has been shown to have direct effects on muscle (Ceglia and Harris, 2012); however, the exact mechanisms remain unknown. The identification of the vitamin D receptor (VDR) on muscle cells (Zanello et al., 1997; Bischoff et al., 2001) provides support for a direct effect of vitamin D on muscle tissue. The functions of vitamin D are characterised as genomic, mediated through the VDR transcriptional effects inside the cell nucleus, and non-genomic, when the VDR induces rapid signalling, situated on the cell membrane and/or cytoplasm. Once vitamin D has been transported to and bound to its nuclear receptor it results in changes in the gene transcription of mRNA and subsequent de novo protein synthesis (Freedman, 1999). Research has demonstrated that vitamin D impacts on both the trans-membranous flows of calcium and phosphate in skeletal muscle, and the synthesis rate of contractile properties (Stewart and Rittweger, 2006).

Vitamin D supplementation (800 IU per day) for periods of 8 to 12 weeks has been reported to reduce postural sway and improve the risk of falling in elderly individuals (Bischoff et al., 2003; Pfeifer et al., 2000). Longer periods (12 months) of vitamin D supplementation (800 IU per day) in the elderly has been shown to increase strength, decrease body sway and increase physical performance (Pfeifer et al., 2009). However, in a healthy elderly population with no vitamin D deficiency, vitamin D supplementation does not appear to improve muscle strength or function (Grady et al., 1991; Johnson et al., 1980). It remains to be seen, whether vitamin D supplementation in healthy persons with no vitamin D deficiency, any enhancement in muscle structural or contractile properties can be attained in the presence of hypo-activity.

1.4.6. Omega-3 (EPA)

Recent studies supplemented healthy young and elderly individuals with ω-3 fatty fish-oils for 8 weeks and found a significant increase in the muscle protein synthetic response to amino acid administration (Smith et al., 2011a; Smith et al., 2011b). Though the mechanism responsible remains to be elucidated, it does not seem to be related to the proposed anti-inflammatory properties of omega-3 fatty acids. Instead, the authors suggested that omega-3 fatty acid supplementation may amplify the stimulatory effects of amino acids on the mTOR/p70S6K signalling pathway. They concluded in the elderly model that ω-3 fatty acids might be useful for the prevention and treatment of sarcopenia (Smith et al., 2011a). Dietary fish oil has also been shown to alleviate soleus muscle atrophy during immobilisation in association with Akt signalling in rats (You et al., 2010). It would therefore, seem reasonable to suggest that more investigation is needed in to the potential of ω-3 fatty acids as a nutritional supplement for attenuating muscle atrophy with hypo-activity. In parallel, it is believed that ω-3 fatty acids may impact on lean body mass though decreasing the effectiveness of catabolic cytokines, reduced protein degradation and improving insulin sensitivity (Siddiqui et al., 2004). There is evidence to suggest that eicosapentaenoic acid (EPA) an ω-3 fatty acid may reduce the pro-inflammatory cytokines associated with inflammation (Magee et al., 2008). Magee et al. (2008) demonstrated *in vitro* that EPA inhibits the effects of tumour necrosis factor alpha (TNF-α) by reducing its apoptotic effects and enabling myogenesis. Previous research has suggested the potential of EPA to increase isometric and isokinetic torque (Houghton and Onambele, 2012). It is unclear whether this supplement would have a beneficial effect during disuse models, where it is generally accepted that there is muscle atrophy (Grosset and Onambele-Pearson, 2008), which is associated with decreased

protein synthesis (de Boer, Selby, et al., 2007), but scant evidence for increased protein breakdown (Ferrando et al., 1996).

1.5. Conclusion

Hypo-activity models result in profound changes in skeletal muscle morphology and strength. Muscle mass and strength losses vary between different hypo-activity models, with immobilisation causing the most profound decreases, greater than bed-rest and limb suspension. Decrements in muscle size and strength are seen in response to hypo-activity models with the greatest decrements seen in antigravity muscles. The decreases in strength seen with hypo-activity models surpass the losses in muscle mass and as such, the nervous system and contractile properties adapt to adjust for this excessive loss of strength. Nutritional supplementation may stand as a viable intervention to combat muscle atrophy with hypo-activity when exercise is not a practical prescription. There are several potential nutritional supplements that could be used to combat muscle atrophy but extensive research is needed to determine the most affective.

1.6. Aims and objectives

Consequently, the overall aim of the current thesis was to determine the role that nutritional supplementation may play in attenuating hypo-activity-induced atrophy. More specifically, the objectives were:

1. To analyse changes in *in vivo* muscle as well as endocrine parameters and blood flow kinetics in response to immobilisation
2. To determine the impact of several different nutritional supplements on the magnitude of change in these physiological parameters
3. To identify whether pre-immobilisation supplementation has any impact on the effectiveness of reducing immobilisation-induced atrophy

1.7. Overview of thesis

Chapter 1: This chapter has already discussed the varying impact of different hypo-activity models on:

- muscle morphology
- muscle strength
- muscle fatigability

The chapter also discussed potential nutritional interventions for preventing the loss of muscle mass/function seen with hypo-activity.

Chapter 2: Effects of essential amino acid supplementation on muscular adaptations to 3 weeks of combined unilateral glenohumeral & radiohumeral joints immobilisation

Participants

Females n = 11, males n = 5, aged 21 ± 3.1 years

Intervention

Non-dominant arm immobilised in a sling for 9 waking hours a day over 3 continuous weeks

Essential amino acid vs. placebo supplementation during immobilisation

Measures

- Muscle and sub-cutaneous adipose thickness (b-mode ultrasonography)
- Upper and lower arm girth (anthropometry)
- Isometric torque (dynamometry)
- Muscle activation (electromyography)
- Serum interleukin-6 (ELISA)

Chapter 3: Omega-3 fatty acids and vitamin D in immobilisation: Modulation of appendicular mass content, composition and structure

Participants

Females n = 15, males n = 9, 23.0 ± 5.8 years

Intervention

Non-dominant arm immobilised in a sling for 9 waking hours a day, over 2 continuous weeks

Placebo vs. omega-3 fatty acids vs. vitamin D supplementation during immobilisation

Measures

- Muscle and sub-cutaneous adipose thickness (B-mode ultrasonography)
- Body composition (DXA)
- Arm girth (anthropometry)

Chapter 4: Omega-3 fatty acids and vitamin D in immobilisation: Modulation of muscle functional, vascular and electromyographic profiles

Participants

Females n = 15, males n = 9, 23.0 ± 5.8 years

Participants were the same sample as in Chapter 3

Intervention

Non-dominant arm immobilised in a sling for 9 waking hours a day, over 2 continuous weeks

Placebo vs. omega-3 vs. vitamin D supplementation during immobilisation

Measures

- Isometric and isokinetic torque (dynamometry)
- Muscle co-contraction (electromyography)
- Muscle fatigability indices (dynamometry and electromyography)
- Arterial resting blood flow (Doppler ultrasound)

Chapter 5: Systemic endocrine profile following a small muscle, short duration, limb immobilisation

Participants

Females n = 12, males n = 9, 22.7 ± 6.7 years

Participants were the same sample as in Chapter 3, with the exception of three participants for whom blood samples were not obtained

Intervention

Non-dominant arm immobilised in a sling for 9 waking hours a day, over 2 continuous weeks

Placebo vs. omega-3 vs. vitamin D supplementation during immobilisation

Measures

- Circulating creatine kinase (CK)
- Circulating interleukin 6 (IL-6)
- Circulating interleukin 10 (IL-10)
- Circulating tumour necrosis factor alpha (TNF-α)
- Circulating insulin like growth-factor (IGF-1) concentrations

Chapter 6: Impact of pre-immobilisation protein supplementation on the time course of immobilisation-induced atrophy and asthenia

Participants

Females n = 6, males n = 4, 22.1 ± 4.3 years

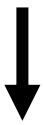
Intervention

Non-dominant arm immobilised in a sling for 9 waking hours a day, over 2 continuous weeks

Essential amino acid vs. placebo supplementation before immobilisation

Measures

- Muscle and sub-cutaneous adipose thickness (B-mode ultrasonography)
- Body composition (DXA)
- Arm girth (anthropometry)
- Isometric and isokinetic torque (dynamometry)
- Muscle co-contraction (electromyography)
- Muscle fatigability indices (dynamometry and electromyography)
- Arterial resting blood flow (Doppler ultrasound)



Chapter 7: Summary, conclusions and recommendations

- Contains the general discussion and conclusion of the thesis
- In combining the findings of the preceding chapters, this chapter also considers the implications of these for future research in addition to outlining possible future directions

Chapter 2: Effects of essential amino acid supplementation on muscular adaptations to 3 weeks of combined unilateral glenohumeral & radiohumeral joints immobilisation

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2.1. Introduction

As discussed in Chapter 1, skeletal muscle has adaptive potential, meaning it is capable of altering its structure in response to environmental changes. In other words, the human muscular system shows great plasticity in response to different levels of physical activity. Prolonged reductions in muscle activity and mechanical loading, such as those experienced during limb suspension (de Boer, Maganaris, et al., 2007), bed-rest (LeBlanc et al., 1992), and immobilisation (Veldhuizen et al., 1993), result in numerous physiological adaptations in skeletal muscle structure and function. It has consistently been demonstrated that long periods of hypo-activity result in skeletal muscle atrophy (Abe et al., 1997; Akima et al., 2001) and a decrease in maximal voluntary strength (Clark et al., 2008; Hortobagyi et al., 2000; Miles et al., 1994; Semmler et al., 2000; Veldhuizen et al., 1993; Yue et al., 1997). However, discrepancies exist between the reductions in muscle strength and in muscle size seen with hypo-activity models (Clark et al., 2006; Kawakami et al., 2001; Thom et al., 2001; Veldhuizen et al., 1993). These observations would tend to suggest that part of the observed reduction in maximal voluntary contraction (MVC) may be due to reduced drive from the central nervous system to the muscle.

Reductions in muscle mass with hypo-activity may be the result of a decrease in protein synthesis, an increase in protein breakdown or a combination of the two (Bamman et al., 1998). On the topic of potential for increased protein breakdown, it is noted that periods of immobilisation have been associated with increased fatty tissue deposition (Manini et al., 2007). Adipose tissue produces and secretes inflammatory cytokines, for example interleukin-6 (IL-6) (Mohamed-Ali et al., 1998) and the expression and plasma levels of such cytokines increase with increased adiposity (Ahima and Flier, 2000; Pedersen et al., 2003). The

elevation of “pro-inflammatory” cytokines such as IL-6 is generally viewed to be potentially deleterious with regard to skeletal muscle. For example, it has been observed that IL-6 either directly or indirectly mediates catabolic effects on skeletal muscle (Goodman, 1994). It should be noted that whilst some IL-6 signalling is also important to initiate the cascade to signal protein synthesis (Saini et al., 2009), findings from Haddad et al. (2005) suggest that down-regulation of growth factor-mediated intracellular signalling may be a mechanism contributing to the development of muscle atrophy induced by significantly elevated IL-6. On the topic of potential for increased protein breakdown, it is noted that Ferrando et al. (1996) reported a loss of lean muscle mass, accompanied with a 14 % decrease in protein synthesis and no change in protein breakdown in response to 14 days simulated microgravity. Gibson et al. (1987) noted a marked fall in muscle protein synthesis in response to 7 weeks leg immobilisation. More recently, a shorter period of immobilisation (21 days) provided very little evidence of any increases in messenger ribonucleic acid (mRNA) for catabolic enzymes or increases in enzyme activity during this period (de Boer, Selby, et al., 2007). In contrast to this there is some suggestion in the literature that such increases in catabolic potential do occur, and for this event to happen very quickly (48 hours) after immobilisation (Urso, Scrimgeour, et al., 2006). Nonetheless, the weight of the evidence would suggest that it is unlikely that protein breakdown is a key modulator in the process of muscle atrophy occurring during immobilisation in humans (Rennie, 2009; Rennie et al., 2010).

There is clear evidence that physical activity (and resistance exercise in particular) provides an anabolic stimulus during hypo-activity (Akima et al., 2000; Bamman et al., 1998; Ferrando et al., 1997). Moderate-resistance exercise (5 sets x 8 reps to volitional fatigue) every other day during 14 days bed-rest prevented a decline in muscle protein synthesis, whereas bed-rest alone elicited a 46 %

decrease in muscle protein synthesis (Ferrando et al., 1997). Isometric leg press training (3 sets x 30 reps) during 20 days of bed-rest resulted in no changes in physiological cross sectional area (PCSA), whereas in the bed-rest control group PCSA decreased by 7.8 % (Akima et al., 2000). A number of nutritional interventions exist that have proven anabolic effects. For instance, when supplemented with either whole proteins (Burke et al., 2001) or simply essential amino acids (EAA) (Brooks et al., 2008), the benefits of exercise appears additive. It may not, however, always be practical to prescribe exercise to counteract the atrophy brought about by hypo-activity. This would tend to be linked to the presence of counter-indications for exercise such as pain or immobilisation in a cast. In these cases, supplementing the diet with potential/recognised hypertrophic nutrients may be an intervention of choice for preventing the loss of muscle mass/function seen with hypo-activity. Promising results have been observed in research utilising an increased protein intake (from 0.6 to 1.0 $g \cdot kg^{-1} \cdot day^{-1}$) to ameliorate the catabolic responses to 1 week of bed-rest (Stuart et al., 1990).

Unlike protein supplementation, EAA supplementation does not affect satiety, and further, does not alter the metabolic effects of subsequent meals (Paddon-Jones et al., 2005). This thus, means that EAAs are likely to be the preferred nutritional supplement. Bolus oral ingestion of EAAs produces a rapid, several-fold increase in plasma amino acid levels (Paddon-Jones, Sheffield-Moore, Zhang, et al., 2004) and has been shown to stimulate net protein synthesis to a greater extent than a mixed meal or a solution containing nonessential amino acids (Tipton et al., 1999). A study has in fact suggested that providing a nutritional supplement enriched with EAAs could improve lean body mass, strength and physical function, even without exercise (Katsanos et al., 2006).

It is evident from previous research that immobilisation decreases lean body mass, and that EAA supplementation improves lean body mass in several models from increased physical activity (Kraemer et al., 2009) to bed-rest (Paddon-Jones, Sheffield-Moore, Zhang, et al., 2004). Whilst these findings are interesting, they cannot be extended to all models of hypo-activity. Indeed, previous studies show that each model of hypo-activity impacts on muscle structure and function differently (Clark, 2009), hence, decreasing one's ability to extrapolate the effects of one treatment between models of hypo-activity. Research is lacking in immobilisation studies in humans in which EAA supplementation is given as an intervention.

The aim of the present study was, therefore, to determine the role that EAA supplementation may play in attenuating atrophy induced through a model that would emulate relatively short-term decreased local mobility/activity in humans. The differential effect of immobilisation on *in vivo* muscle (size, strength), sub-cutaneous adipose tissue (thickness), neural (electromyography (EMG) activity) and cytokine (circulating interleukin-6) factors, in the presence or absence of EAA supplementation, was systematically monitored. It was hypothesised that EAA supplementation during combined shoulder and arm immobilisation would attenuate the deleterious changes in muscle, fat and cytokine *in vivo* characteristics associated with hypo-activity models.

2.2. Methods

2.2.1. Participants

Eighteen healthy volunteers were recruited from the local university campus; via word of mouth and posters. All participants gave written informed consent to take part in the study. Prior to commencement of the study, the local Ethics Committee of Manchester Metropolitan University approved all procedures and experimental protocols. Exclusion criteria included any conditions requiring the use of medications likely to affect muscle function or musculoskeletal health, and any current or history of kidney/liver disease. Before taking part in the study, participants completed a questionnaire (Appendix 4) to ascertain health and habitual physical exercise levels. The questionnaire confirmed that all participants were recreationally active and free from recent upper limb injury. Participants were randomly assigned to one of two groups, though two subsequently withdrew during the course of the study for personal reasons. Hence, the present report describes data from the remaining sixteen participants: EAA group [EAA: n = 9 (3 male, 6 female), 20 ± 1.5 years, 63.8 ± 8.1 kg, 169.6 ± 8.7 cm] and placebo group [PLA: n = 7 (2 male, 5 female), 23 ± 3.9 years, 66.9 ± 9.5 kg, 167.0 ± 7.2 cm].

2.2.2. Study design

The study used a randomised, double-blind, placebo-controlled design. Following familiarisation with testing procedures at least one week prior to testing, participants attended the laboratory between 8h00 and 10h00 after an overnight fast, as to standardise the time of day across sessions and participants. The non-dominant arm of the participants was placed in a sling immediately after baseline testing and the correct procedure for sling wear was explained to the participant. Essentially, the aim was to minimise any movement medio-laterally at the elbow

and shoulder (see Figure 2.1), whilst requiring participants to not contract the upper musculature (including the hands) during the hours of immobilisation. Participants were required to wear this sling for approximately nine waking hours per day, over a 3-week period. A 3-week period was chosen as it is in-line with previous upper limb studies as described in Chapter 1. Removal of the sling was only permitted when necessary (e.g. taking a bath/showering, driving, going to bed at night) but the 9 hours did not have to be continuous. Measures of muscle and subcutaneous adipose thickness, upper and lower arm girth, serum cytokine, isometric torque and electromyographic activation were taken immediately before immobilisation (Pre; i.e. on day 1 of immobilisation) and immediately on removal of the sling (Post; i.e. within 2 days of remobilisation). Details of all the procedures are below.

During the 3 weeks immobilisation phase participants in the EAA group ingested 2 x 30 mL per day of a commercially available EAA drink (BodyFortress, Holland & Barrett, UK), whereas participants in the PLA group ingested a placebo drink. Each 30 mL dose of the *Liquid Amino* nutritional supplement is described by the manufacturer as containing 22 g of protein (~30% EAA) i.e.: 1121 mg L-Alanine; 1223 mg L-Arginine; 655 mg L-Aspartic Acid; 1456 mg L-Glutamic Acid; 3394 mg Glycine; 1311 mg L-Histidine; 218 mg L-Hydroxylysine; 1791 mg L-Hydroxyproline; 175 mg L-Isoleucine; 378mg L-Leucine; 480 mg L-Lysine; 131 mg L-Methionine; 233 mg L-Phenylalanine; 1995mg L-Proline; 495 mg L-Serine; 277 mg L-Threonine; 87 mg L-Tyrosine; and 320 mg L-Valine. The placebo mixture consisted of a homemade blend of water, wild cherry flavouring, honey, salt and a red food colourant, designed to emulate the appearance and to a degree the taste of the EAA supplement. At day 1 of immobilisation, participants were given clear instructions regarding the dosage of the formula. Participants ingested the twice-daily dose of EAA or placebo at 8h00 and 18h00, these timings allowed for

consistency within and between participants. During the 3 weeks immobilisation period, participants were instructed to maintain their normal diet and refrain from unaccustomed strenuous activity. To monitor this, participants completed a daily food and activity diary and wore a pedometer to record the number of steps taken each day. Participants were asked to report their dietary intake as accurately as possible and were provided with strict instructions. Dietary analysis does rely on the compliance of participants and this cannot be guaranteed, however, previous studies have reported good reliability with self-reported dietary intake (Brunner et al., 2001; Day et al., 2001).

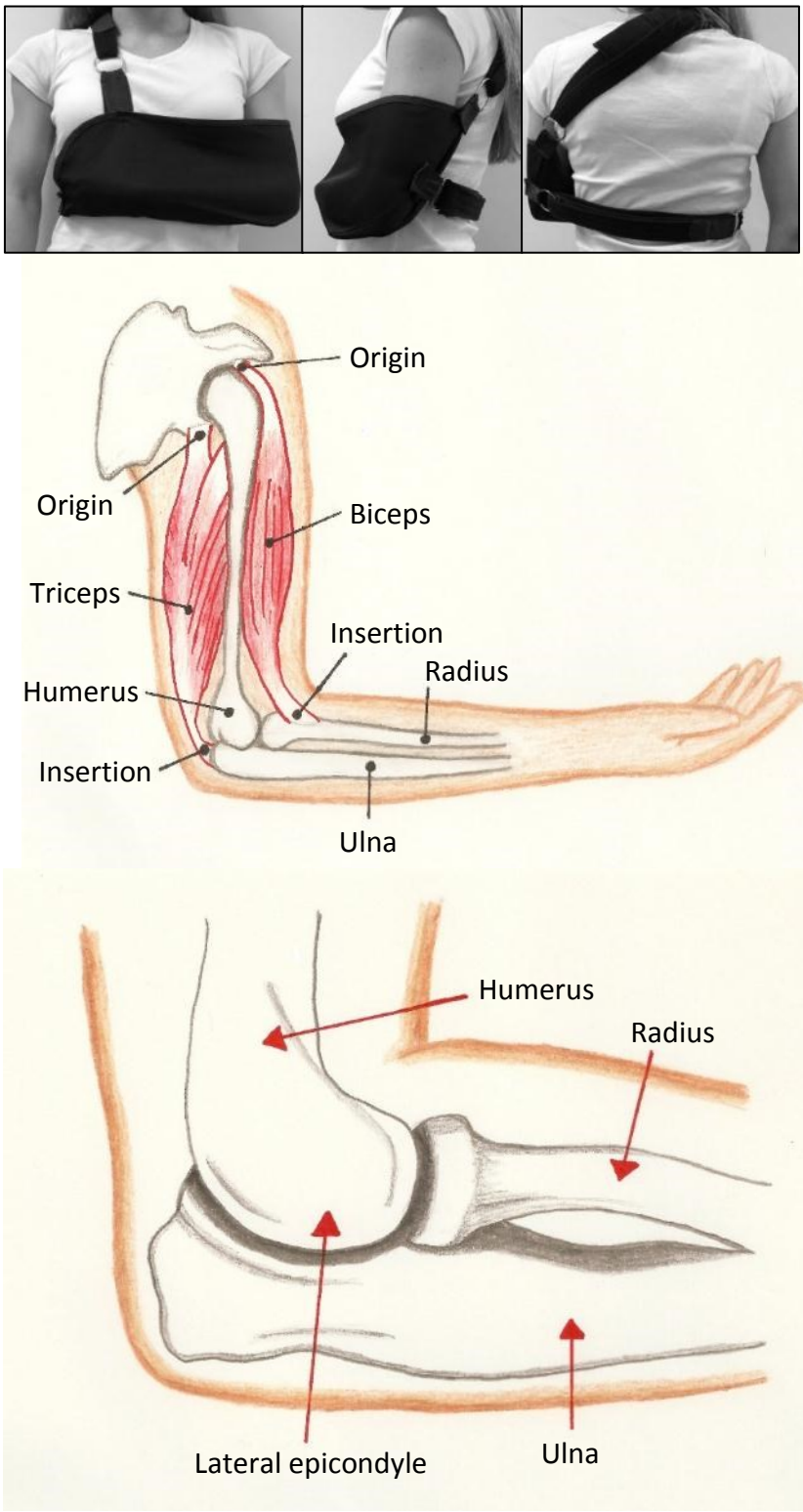

Figure 2.1. (A) Front, side and back view demonstrating sling wear. The sling was initially demonstrated by the experimenter and on subsequent applications, sling application was self-administered by each participant. (B) Anatomical diagram of the immobilised muscles and bones. (C) Anatomical diagram of the radiohumeral joint.

2.2.3. Muscle and sub-cutaneous adipose thickness measures

To avoid fluid shifts that might induce interstitial and/or intracellular changes all images were recorded after approximately 20 minutes of seated rest (Berg, Tedner, et al., 1993). B-mode ultrasonography (AU5, Esaote, Genoa, Italy), using a 7.5-MHz linear phased-array probe (image depth: 37.1 – 92.8 mm), was applied in the sagittal plane to obtain images of the muscles and sub-cutaneous adipose tissue of the upper arm. Images were recorded using an analogue capture card (Pinnicale DV500, Adobe, Maidenhead, UK) and stored for later analysis. Care was taken to apply minimal pressure onto the tissue area of interest during scanning, in order to avoid any image distortion. This method has been used many times previously by others, with great reliability (Miyatani et al., 2004; Miyatani et al., 2002; Onambele et al., 2006).

Images were obtained with the participant in an upright-seated position, their arms hanging by the sides in the anatomical position. In the upper arm, the proximal and distal insertions of the biceps and triceps brachii were identified, marked on the skin and the mid-point was identified. Upper arm ultrasonography images were collected in the sagittal plane, mid-limb length, and at the level of the mid-acromiale-radial. Muscle thickness was measured as the distance from the top of the superficial muscle aponeurosis to the bone in the biceps and triceps brachii (see Figure 2.2). Sub-cutaneous adipose thickness was measured as the distance from the bottom of the epidermis to the top of the superficial muscle aponeurosis in the biceps and the triceps brachii (see Figure 2.2). These distances were measured at three standardised points on each ultrasound frame to obtain average muscle and sub-cutaneous adipose thicknesses using ImageJ analysis software (ImageJ 1.37, Maryland, USA).

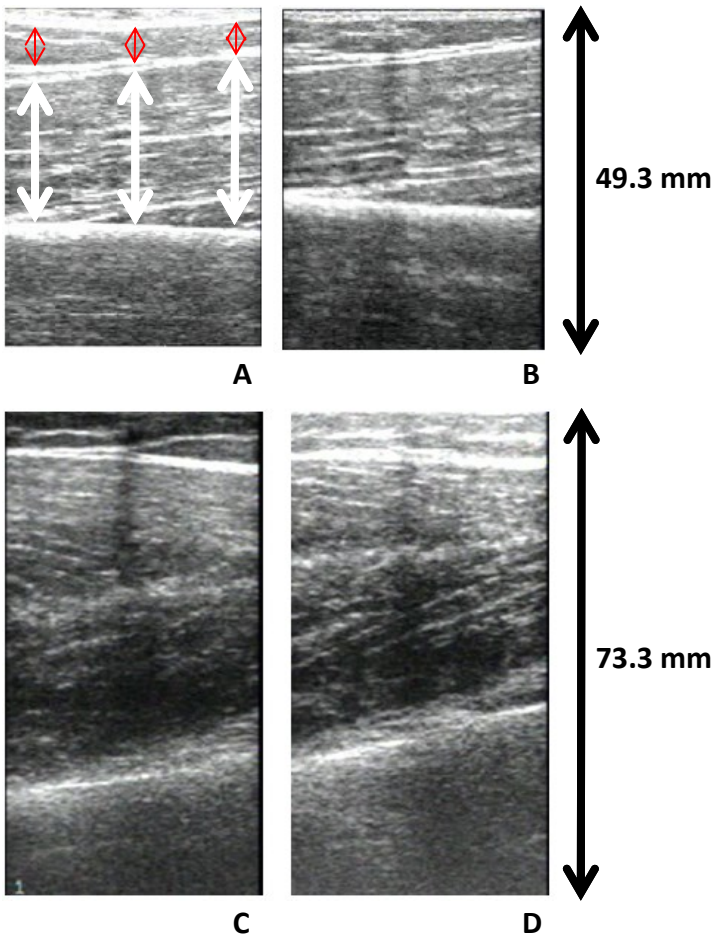

Figure 2.2. Ultrasound images of the biceps Pre- (A) and Post-immobilisation (B) and the triceps brachii Pre- (C) and Post-immobilisation (D). The red arrows indicate sub-cutaneous adipose thickness measurement sites. The white arrows indicate muscle thickness measurement sites.

2.2.4. Arm girths

Participants were asked to assume a relaxed standing position with arms hanging by the sides, palms facing the sides of the hips. Upper arm girth (relaxed) was measured using a measuring tape at the level of the mid-acromiale-radial: with the arm abducted slightly to allow the tape to be passed around. Forearm girth was measured at a fixed point 1/4 of the way (from the proximal end) along the length of the radiale-stylion: with the arm slightly flexed at the shoulder and the elbow extended.

2.2.5. Isometric dynamometry

Maximal isometric torque was measured using a Cybex dynamometer (Cybex, New York, USA). Participants were positioned as per the manufacturers' recommendations. Briefly, they were fastened to the dynamometer in a supine position with the trunk and lower limbs firmly strapped to minimise any extraneous movement. The axis of rotation of the dynamometer was aligned with the anatomical axis of rotation of the elbow joint (lateral epicondyle) and the upper arm secured using padded strapping, mid acromiale-radial. The participant's functional range of motion was measured and safety stops were set both electronically and manually, to prevent hyper- extension/flexion. A gravity correction was made for limb weight on torque measurements. All measurements were preceded by a warm-up period consisting of 2 mins of isokinetic contractions increasing in intensity, followed, after 2 mins rest, by one isometric contraction at a 90° elbow joint angle. Following a further 2 mins rest, two repetitions of isometric contractions, 60 sec apart, were performed at a 90° elbow joint angle and the highest torque was recorded as the participant's MVC. During all MVC attempts, participants were instructed to rapidly exert maximal torque against the Cybex lever arm over a 3-4 s period, first in the direction of flexion and, 5 sec after return to baseline, in the direction of elbow extension. Participants were encouraged to exert maximal torque through a combination of verbal and visual feedback. Torque and angle were displayed on the screen of a computer (Macintosh G4; Apple Computer, Cupertino, CA), which was interfaced with an A/D system (Acknowledge, Biopac Systems, Santa Barbara, CA) with a sample frequency of 200 Hz. Peak torque was averaged over a 500 ms period (i.e. 250 ms either side of the instantaneous peak). The highest of the repeated efforts was used as the participant's measure of MVC.

2.2.6. Electromyographic measurement

EMG was used to asses muscle activation patterns. The skin was prepared by shaving, abrading and cleaning with an alcohol-wipe to minimise resistance below 5kΩ. Self-adhesive Ag-Cl (Medicotest, Rugmarken, Denmark) surface electrodes were then placed in pairs on the midsagittal plane of the biceps and the triceps brachii muscles, with reference electrodes placed on the lateral and medial epicondyle of the humerus. Cross talk was minimised by a) the cables being insulated, b) having very small electrodes c) leaving a 2 cm gap between electrodes (Winter et al., 1994). These data were acquired using the same system that acquired the torque data (as described above). Raw EMG data were recorded at 2000 Hz, with a band pass filter set at 10-500 Hz, with a notch set at 50 Hz.

2.2.7. Interleukin-6 (IL-6)

After a 10-12-hour overnight fasting period, before any of the other laboratory measures, blood samples were taken from the antecubital vein of the forearm by a hospital-trained phlebotomist, using a 21ml gauge needle (S-Monovette, Sarstedt, Germany). Subsequently, 5-10 mL of venous blood was permitted to clot on ice for up to 1 hour. Samples were then centrifuged (Hermle Z 380, Huddersfield) at 5°C and at 7500 rpm for 15 minutes to separate the serum from the blood cells. Aliquots (~1000 µl each) of the resulting sera were stored at -20^{o}C for later analysis. IL-6 (R&D systems, Abingdon, UK) in the sera samples was quantified using standard quantikine high sensitivity (i.e. up to 10 pg/mL of IL-6) enzyme-linked immunosorbent assay (ELISA). Optical density was read at 490 nm with a lambda correction at 650 nm. Intra-assay (coefficient of variation (CV) = 7.4 %) and inter-assay (CV = 7.8 %) precisions, were all within acceptable boundaries. The minimum detectable dose ranged from 0.016 to 0.110 pg/mL (average = 0.039 pg/mL).

2.2.8. Statistics

Data were analysed using IBM SPSS v19 (IBM Inc, USA). The Shapiro-wilk test revealed some of the data to be non-parametric (triceps sub-cutaneous adipose thickness, triceps muscle thickness change, biceps torque, biceps torque change, EMG and serum IL-6) and in those cases, the data sets were log transformed. Once transformed the data was checked for normality. Parametric Pre to Post difference data were analysed using paired sample t-tests. If non-parametric, the differences between Pre to Post were analysed using the Wilcoxon signed-rank test. Between group differences in relative change data were analysed either using unpaired t-tests (if parametric), or Mann-Whitney U test (if non-parametric). All data are presented as mean ± standard deviation (SD). Statistical significance was set with alpha at ≤0.05.

2.3. Results

2.3.1. Measurements reliability

The assessment of the intra- and inter-day repeatability of measurements was conducted in a sub-sample of five participants. Those randomly chosen participants were asked to attend a separate repeatability testing date for between day measures, no less than seven days prior to their Pre-immobilisation testing session. A seven-day period was chosen as to not let the testing session impact on the results of the sub-sequent session. The repeatability of unilateral isometric strength, EMG, muscle and sub-cutaneous adipose thickness, as well as anthropometry were measured at the same time-of day to avoid any difference owing to diurnal effects (Pearson and Onambele, 2005). Within-day CV of 4.9 %, 4.9 %, 3.6 %, 8.9 %, 0.6 % and 4.4 %, and between-day CVs of 8.6 %, 8.6 %, 4.0 %, 7.4 %, 1.7 % and 6.4 % were yielded for unilateral elbow flexion torque at 90°,

unilateral elbow extension torque at 90°, mid-upper arm biceps muscle thickness, mid-upper arm triceps muscle thickness, mid-upper arm biceps fat thickness and mid-upper arm triceps fat thickness, respectively.

2.3.2. Daily physical functioning and nutritional intake

No significant change was observed in habitual physical activity ($p>0.374$) or in calorific intake ($p>0.420$) during the course of immobilisation. This effect was true for both the EAA and the PLA groups. Dietary analysis revealed no significant difference in habitual protein intake over the three-week period between the EAA and PLA groups (see figure 2.3).

2.3.3. Muscle and sub-cutaneous adipose thicknesses

Muscle and sub-cutaneous adipose thickness values are displayed in Table 2.1. Comparisons of percentage change in biceps and triceps muscle thicknesses revealed no significant differences between the EAA and PLA groups (biceps $p=0.096$; triceps $p=0.172$). Comparison of percentage change in biceps sub-cutaneous adipose thickness revealed a significant difference between the EAA and PLA groups (Student's t-test; $p=0.03$). Comparison of percentage change in triceps sub-cutaneous adipose thickness revealed no significant difference between the EAA and PLA groups (Student's t-test; $p=0.100$).

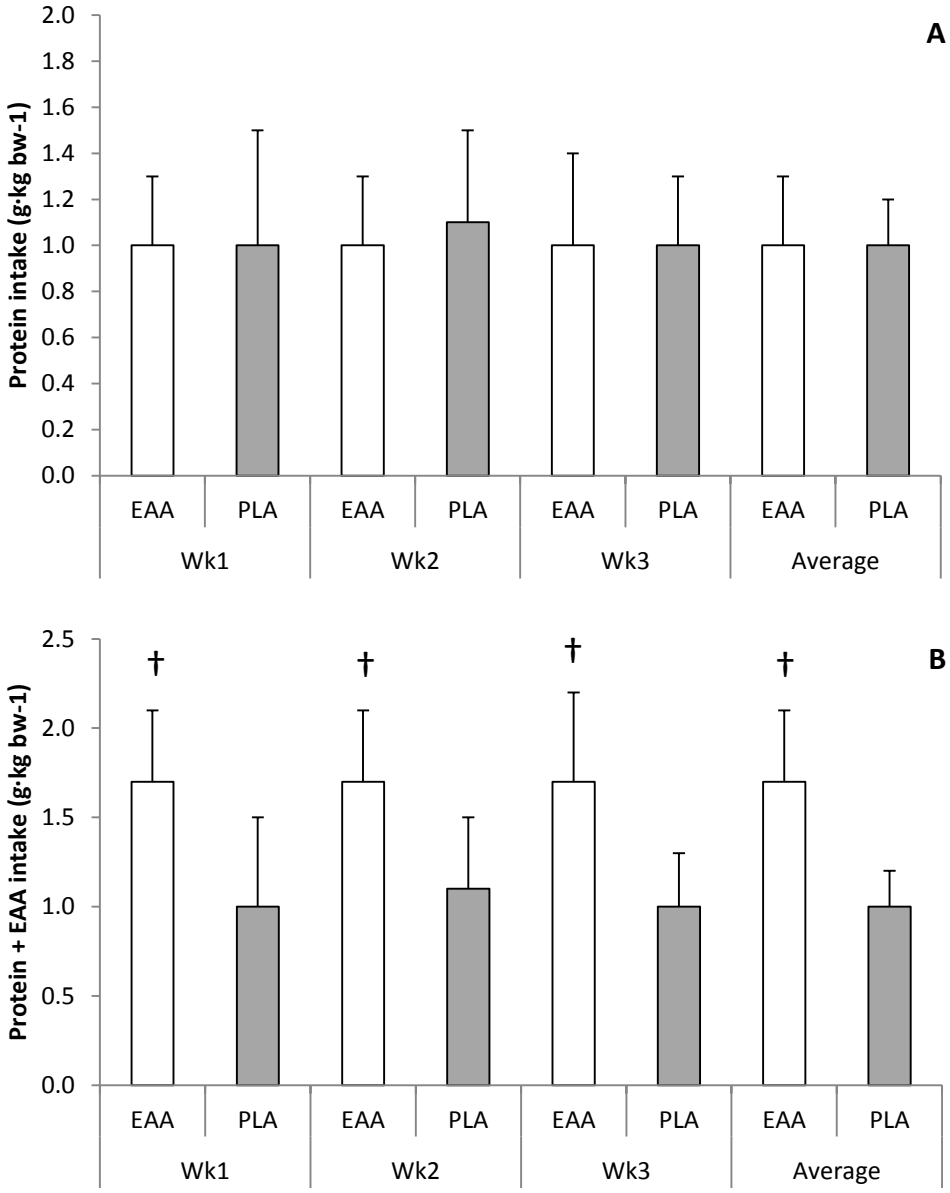

Figure 2.3. Daily protein intake (g·kg bw^{-1} ± SD) for: A) the EAA group (white bars) and PLA group (grey bars); B) EAA group plus protein intake provided by the EAA supplement (white bars) and the PLA group (grey bars). † Significant difference in protein intake between supplement groups.

Table 2.1. Differences in muscle and sub-cutaneous adipose thickness measurements Pre- and Post-immobilisation (mm ± SD) of the biceps and the triceps brachii. Values are reported for the EAA and PLA groups. Percentage changes from Pre- to Post-immobilisation are also reported (% change ± SD). * Significantly different change from Pre- to Post-immobilisation ($p<0.05$). † Significant difference in % change between EAA and PLA groups.

		EAA	PLA
Biceps muscle thickness	Pre	24.7 ± 2.5	25.2 ± 4.2
	Post	23.4 ± 3.3	25.2 ± 4.6
	% Change	-5.1 ± 11.4 %	-0.4 ± 3.8 %
Biceps sub-cutaneous adipose thickness	Pre	5.0 ± 3.0	4.9 ± 3.7
	Post	4.9 ± 3.0	5.0 ± 2.7
	% Change	-3.3 ± 11.8 % †	19.6 ± 30.5 % †
Triceps muscle thickness	Pre	32.0 ± 7.9	30.4 ± 7.5
	Post	27.8 ± 7.0*	28.4 ± 6.5
	% Change	-11.7 ± 15.0 %	-5.5 ± 11.4 %
Triceps sub-cutaneous adipose thickness	Pre	10.3 ± 6.0	13.0 ± 7.9
	Post	10.1 ± 5.4	13.0 ± 8.2
	% Change	-0.8 ± 9.3 %	0.0 ± 11.4 %

2.4.4. Arm girth

Pair-wise (Students t-test) comparisons revealed that upper and lower arm girths decreased significantly Post-immobilisation in the PLA group (30.6 ± 3.6 cm to 30.1 ± 3.6 cm ($p<0.01$) and 25.9 ± 1.9 cm to 25.4 ± 1.8 cm ($p<0.01$), respectively) but not the EAA group (29.3 ± 3.3 cm to 29.2 ± 3.2 ($p>0.05$) and 25.0 ± 1.8 to 24.9 ± 1.8 ($p>0.05$), respectively). Percentage change in arm girth was significantly greater in the PLA group than the EAA group in both the upper and lower arm (Student's t-test; $p=0.010$, $p=0.045$, respectively) (Figure 2.4).

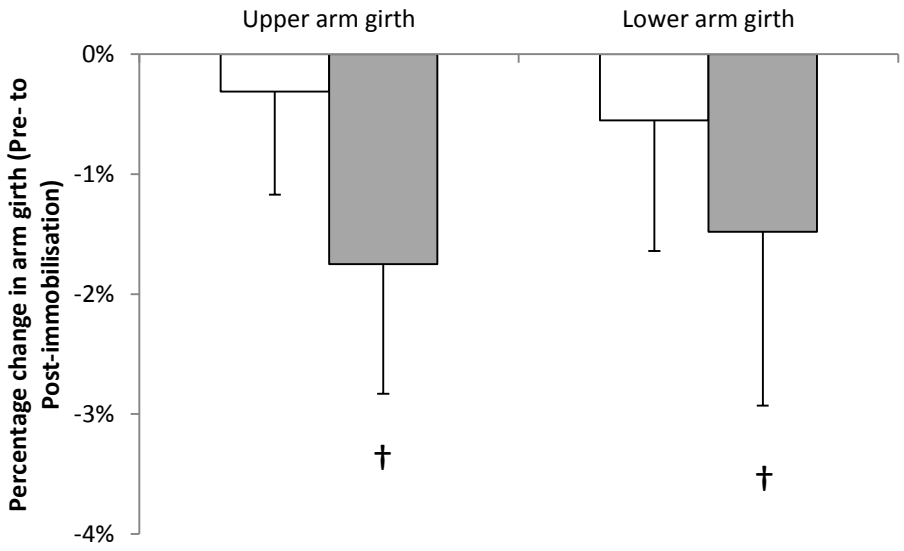

Figure 2.4. Percentage changes (% ± SD) in upper and lower arm girth for the EAA (white bars) and the PLA (grey bars) groups. † Significant difference in % change between EAA and PLA groups.

2.3.5 Isometric dynamometry

Elbow flexion torque values are shown in Table 2.2. Pair-wise (Students t-test) comparisons revealed elbow flexion torque decrease from Pre- to Post-immobilisation only in the EAA group ($p=0.04$). Elbow extension torque values are shown in Table 2.2. Despite the apparent trend for a decrease in elbow flexion and

extension torque values, to be greater in the PLA group than EAA group, this effect was not significant ($p>0.05$).

Normalising elbow flexion torque by biceps muscle thickness, this ratio decreased by 6 % in the EAA group (0.81 Nm/mm (Pre) to 0.76 Nm/mm (Post)) and 20 % in the PLA group (0.80 Nm/mm (Pre) to 0.64 Nm/mm (Post)) ($p>0.05$). Normalising elbow extension torque by triceps muscle thickness, this ratio increased by 15 % in the EAA group (0.60 Nm/mm (Pre) to 0.69 Nm/mm (Post)) and decreased by 23 % in the PLA group (0.65 Nm/mm (Pre) to 0.50 Nm/mm (Post)) ($p>0.05$).

2.3.6. Agonist and antagonist EMG activity

EMG data were non-parametric. Values for agonist EMG activity and antagonist co-contraction ratios are shown in Table 2.2. Statistical analysis revealed no within or between group differences in agonist EMG activity or antagonist co-contraction ratios in either flexion or extension isometric contractions.

2.3.7. Circulating IL-6 levels

Changes in IL-6 are shown in Figure 2.5. A pair-wise (Students t-test) comparison revealed no significant effect of supplement group on the degree of percentage change in IL-6 values from Pre- to Post-immobilisation ($p>0.05$).

Table 2.2. Differences in elbow flexion and extension torque (Nm ± SD), agonist activation (mV ± SD) and co-activation ratio (% ± SD) Pre- and Post-immobilisation. Values are reported for the EAA and PLA groups. Percentage change in mean torque and agonist EMG activity from Pre- to Post-immobilisation are also reported. * Significantly different change from Pre- to Post-immobilisation ($p<0.05$).

		EAA	PLA
Elbow flexion Torque	Pre	20.0 ± 7.0	20.2 ± 5.3
	Post	17.7 ± 7.0* (p=0.04)	16.2 ± 2.7 (p=0.21)
	% Change	-11.5 %	-19.8 %
Elbow flexion agonist EMG activity	Pre	173 ± 176	170 ± 71
	Post	236 ± 73	207 ± 153
	% Change	36 %	21 %
Elbow flexion antagonist co-activation ratio	Pre	69.1 ± 23.0 %	66.5 ± 34.7 %
	Post	54.3 ± 13.8 %	53.1 ± 13.1 %
Elbow extension torque	Pre	19.3 ± 5.2	19.8 ± 4.6
	Post	19.1 ± 7.7 (p=0.82)	14.3 ± 6.6 (p=0.20)
	% Change	-1.0 %	-27.8 %
Elbow extension agonist EMG activity	Pre	281 ± 112	252 ± 172
	Post	156 ± 14	289 ± 118
	% Change	-44.5 %	14.7 %
Elbow extension antagonist co-activation ratio	Pre	60.1 ± 20.7 %	81.4 ± 23.9 %
	Post	59.4 ± 18.6 %	64.5 ± 21.4 %

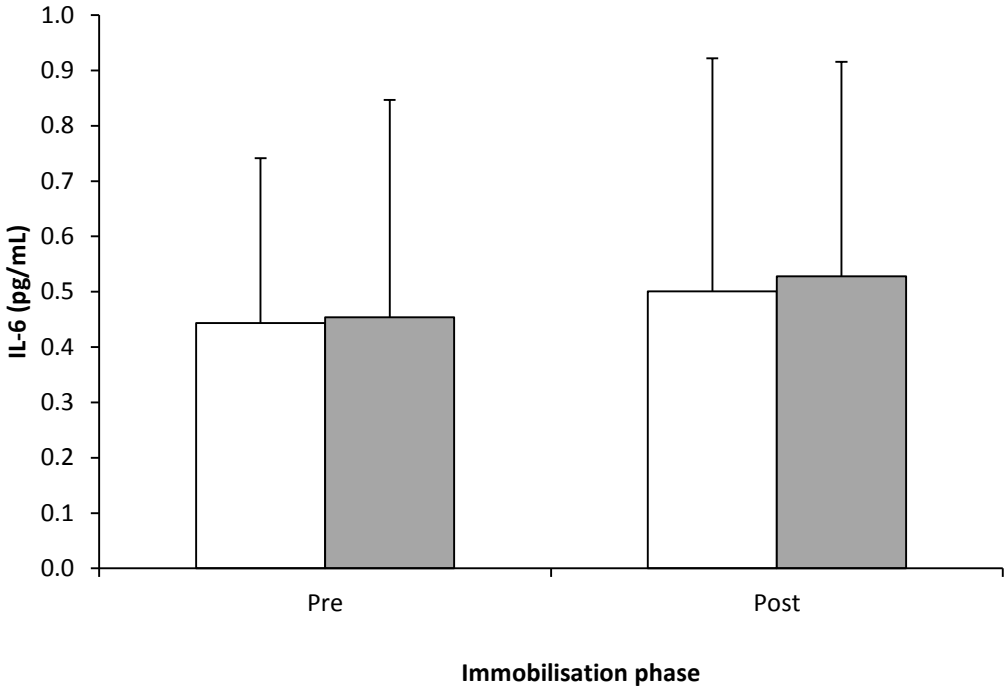

Figure 2.5. Plasma IL-6 levels for the EAA (white bars) and the PLA (grey bars) groups, Pre- and Post-immobilisation (pg/mL ± SD).

2.3.8. Association studies

Bivariate correlations between relative change values for all reported measures are displayed in Table 2.3. The change in bicep sub-cutaneous adipose thickness correlated with the change in triceps brachii muscle thickness (r=0.57, p=0.01). The relative change in upper arm girth correlated with percentage changes in lower arm girth (r=0.70, p=0.001), elbow extension torque (r=0.52, p=0.03), and IL-6 levels (r=0.56, p=0.02). Percentage changes in extension torque and IL-6 levels were also strongly correlated (r=0.80, p<0.001).

Table 2.3. Bivariate correlations among percentage changes in the outcome measures. Correlations marked with * = p<0.05, ** = p<0.01, *** = p<0.001.

Variables	1	2	3	4	5	6	7	8	9
1. Biceps muscles thickness	-								
2. Biceps sub-cutaneous adipose thickness	-.09	-							
3. Trcieps muscle thickness	.40	.09	-						
4. Trciesp sub-cutaneous adipose thickness	.24	-.04	**.57***	-					
5. Upper arm girth	-.25	-.14	-.26	.10	-				
6. Lower arm girth	-.17	-.30	-.03	.26	**.70****	-			
7. Flexion torque	-.02	-.28	-.30	-.40	.22	-.05	-		
8. Extension torque	-.24	.32	-.13	-.06	**.52***	.19	.32	-	
9. IL-6 levels	-.42	.31	-.03	.11	**.56***	.28	.08	**.80****	-

2.4. Discussion

The purpose of this study was to determine the effect of 3 weeks of 9-waking-hours-per-day arm immobilisation on *in vivo* muscle characteristics (size, strength and EMG activity), sub-cutaneous adipose content and IL-6 profile and to determine any impact of EAA supplementation on the magnitude of change in these physiological parameters. The hypothesis was that EAA supplementation during arm immobilisation would attenuate the deleterious changes associated with hypo-activity models. Evidence was found to partly support this hypothesis, in that arm girth decreased more so with the placebo supplement than the EAA supplement and with a notable trend towards a greater decrease in elbow flexion and extension torque, as well as significantly further increased sub-cutaneous fatty tissue with the placebo supplement. The changes in muscle thickness (only significant in the triceps brachii), do not explain the differential arm girth responses between groups.

Upper and lower arm girths were shown to significantly decrease Post-immobilisation in the PLA group only. Girth was used as a gross marker of skeletal muscle atrophy, as previously used by Matsumura et al. (2008). Decreases in upper arm girth (0.9 % = 0.04 %/day) suggest a decrease in muscle CSA in the upper arm in the PLA group and this supports previous findings of a decrease in elbow flexor muscles CSA (11.2 % = 0.4 %/day) and volume (11.6 % = 0.4 %/day) with arm immobilisation (Yue et al., 1997). The decrease in lower arm girth (1.1 % = 0.05 %/day) suggests a decrease in forearm muscle CSA in the PLA group. This is in line with findings from Miles et al. (1994) who reported a 4.1 % (0.5 %/day) decrease in forearm muscle CSA with 9 days arm casting. Other studies suggest that forearm immobilisation does not induce a decrease in muscle CSA as evaluated both by magnetic resonance imaging (MRI) and circumference

measurement during relatively short duration (3 weeks) immobilisation (Kitahara et al., 2003; Matsumura et al., 2008). The changes in arm girth, as well as the decrease in upper arm triceps brachii muscle thickness, in the current study suggest that changes in muscle CSA are lower than those reported in previous studies (Miles et al., 1994; Yue et al., 1997). This apparent discrepancy with the rest of the literature, whereby in the current study relatively small degrees of atrophy during limb immobilisation were observed, where others have observed (though not always) a substantial atrophic response could be due to the following: a) the method of measuring the change in muscle CSA: in the present study arm girth and sagittal plane ultrasound scans were used as markers of muscle size, whereas previous studies have used MRI as a direct measure; b) the method of immobilisation and resultant immobilisation compliance: the immobilisation in the present study was for 9 waking hours a day with a self-administered sling, compared to continuous immobilisation in other studies which used casts for the full immobilisation period. Nonetheless, differences in changes in arm girth from Pre- to Post-immobilisation between the EAA and PLA groups suggest that EAA supplementation may attenuate changes in muscle size. This supports promising results observed in 1 week of bed-rest that showed increased protein intake (from 0.6 to 1.0 $g \cdot kg^{-1} \cdot day^{-1}$) to modulate the catabolic response (Stuart et al., 1990).

The significant difference in limb CSA is not reflected entirely in the current ultrasound data, since the current study found no change in biceps muscle thickness but a decrease in triceps brachii muscle thickness Post-immobilisation. The fact that there is a decrease in skeletal muscle tissue with immobilisation, even though relatively moderate in its duration/restriction of movement, was in fact not surprising (Miles et al., 1994). However, it had been expected that the muscle held in the shortened position (i.e. the biceps), to be impacted on more, than the muscle held in the lengthened position (i.e. the triceps brachii). Muscle stretch is a

modulator of sarcomeres being added in series and as such acts as a signal for protein synthesis. Previous research suggests that when the muscle is immobilised in the lengthened position sarcomeres are added in series, and when the muscle is immobilised in the shortened position sarcomeres are lost (Tabary et al., 1972; Williams and Goldspink, 1973). The current findings are in contrast with those of Yue et al. (1997) who investigated the effect of 4 weeks elbow joint immobilisation with a fibre glass cast and reported decreases in elbow flexor CSA (11.2 % = 0.4 %/day) and volume (11.6 % = 0.4 %/day). One probable cause of the discrepancy between the current study and that of Yue et al. (1997), could be the method of quantifying muscle size. Whereas those authors utilised CSA using MRI scans of the whole muscle, the present study used a single plane scan of the biceps at mid-limb length (which does not correspond to mid muscle length). These differences are key, in relation to 1) the fact that the biceps is a fusiform muscle and thus any positional change along its length would result in a difference in size. Since it is recognised that the greatest degree of muscle hypertrophy for a muscle is at the belly (Narici, 1999), it is not surprising that in the present study the degree of change reported is reduced relative to that of those previous authors. 2) the reliability of the single sagittal scans (e.g. CV = 2.2 % in the current study) versus that of CSA (error for repeated measures = <1 %) (Yue et al., 1997), could also be a factor on the ability to capture the real size alteration events in the muscle. It should be noted nonetheless, that a large number of studies have previously found decreases in thigh (0.8 – 1.2 %/day) (Thom et al., 2001; Veldhuizen et al., 1993) and calf (0.3 – 0.6 %/day) (Haggmark and Eriksson, 1979; White et al., 1984) muscle CSA with lower limb immobilisation using a variety of techniques including computed tomography (Haggmark and Eriksson, 1979; Veldhuizen et al., 1993), ultrasonography (Thom et al., 2001) and limb circumference measures (White et al., 1984). Observations are that the degree of

muscle atrophy is greater in the lower limbs than in the upper limbs. This may be explained by the weight-bearing nature and greater musculature in the lower limbs. It may be the case, that the threshold of muscle synthesis is such that it takes little decrease in loading of lower limbs to demonstrate a change in protein metabolism in favour of decreased protein synthesis (Tesch et al., 2008).

Contrary to expectation, no main effect was shown for supplement group on muscle thickness of the biceps or of triceps brachii. This does not support a study that showed a decrease in the catabolic response to 1 week of bed-rest with increased protein intake (Stuart et al., 1990). Stuart et al. (1990) investigated the effects of 7 days bed-rest on two groups consuming iso-caloric diets containing either 0.6 or 1.0 $g \cdot kg^{-1} \cdot day^{-1}$ of protein and found that the decrease in protein synthesis seen with low dietary protein, was prevented by higher dietary protein. However, they did not measure muscle size and the changes found in protein synthesis may not have resulted in phenotypical changes in muscle size. Comparisons between the study of Stuart et al. (1990) and the current study are, therefore, difficult, particularly as their treatment manipulated whole protein content of iso-caloric diets, whereas the current study supplemented the habitual diet with EAAs. The amount of supplement provided in the present study may not have been sufficient to induce an attenuation of muscle thickness decrease. An alternative explanation for the small effectiveness of EAA supplementation in this study can be through the “muscle full” hypothesis (Millward and Pacy, 1995), in which there is an upper limit of amino acid delivery before muscle cells can no longer use them as a substrate for muscle protein synthesis, instead diverting them toward oxidation (Bohe et al., 2001). The mean percentage change in biceps sub-cutaneous adipose thickness was significantly different in the PLA group than in the EAA group, whereby PLA demonstrated an increase in sub-cutaneous adipose thickness from Pre- to Post-immobilisation, and the EAA group

exhibited a slight decrease in sub-cutaneous adipose thickness. This is in agreement with previous studies (Manini et al., 2007). EAA supplementation, therefore, appeared to attenuate the increase in biceps sub-cutaneous adipose thickness. A previous study showed that providing a nutritional supplement enriched with EAAs could improve lean body mass even without exercise (Katsanos et al., 2006). In this case, the results may suggest that the EAA supplement is working towards maintaining lean mass by reducing the increase in fat mass. Indeed levels of circulating insulin help modulate the long-term level of fat stored in the body in any particular environment (Porte et al., 1998; Woods et al., 1998). EAA ingestion has been shown to significantly increase insulin levels (Paddon-Jones, Sheffield-Moore, Zhang, et al., 2004) and, therefore, this may provide an explanation as to why the EAA supplement attenuated the increase in subcutaneous adipose thickness in the current study.

As mentioned in the introduction, previous research suggests that immobilisation results in a greater decrease in muscle strength than muscle size. Miles et al. (1994) investigated the effects of 9 days cast immobilisation suspended from the neck by a sling on the muscles acting on the wrist. They reported a decrease in muscle CSA (4.1 %) and reductions in isometric (29.3 to 32.5 %), concentric (8.9 % to 21.7 %) and eccentric strength (12.5 to 18.5 %) (Miles et al., 1994). A study using casting to immobilise the elbow joint for four weeks found decrements in MVC and a decrease in the maximum load that could be lifted (Yue et al., 1997). Again here, the reduction in MVC force (35 %) was greater than the observed decrease in muscle size (11 %) (Yue et al., 1997). Similarly, in the present study a greater percentage loss in muscle torque (elbow flexion: -11.5 to -19.8 %; elbow extension: -1.0 to -27.8 %) than in muscle thickness (biceps: -0.4 to -5.1 %; triceps brachii: -5.5 to -11.7 %) was generally

found. This therefore, further supports the idea of neural factors being involved in the mal-adaptations to immobilisation.

Elbow flexion torque significantly decreased in the EAA supplement group. Gross ratios of 'isometric strength to muscle thickness' revealed greater decrements in the PLA group compared to EAA group. For the biceps muscle this ratio decreased by 6 % in the EAA group and 20 % in the PLA group. For elbow extension torque by triceps muscle thickness, the ratio in fact increased by 15 % in the EAA group whereas it decreased by 23 % in the PLA group. This is interesting, as it appears that despite a decrease in triceps muscle thickness in the EAA group, the intrinsic quality of the remaining muscle was in fact improved. This may be due to the triceps brachii being immobilised in a lengthened position, allowing sarcomeres to be added in series (Tabary et al., 1972; Williams and Goldspink, 1973) with additional availability of EAAs. These further data explorations tend to support the idea that EAA supplementation may help to attenuate immobilisation-induced decreases in muscle strength.

Data collected for agonist and antagonist EMG activity highlighted no differences in agonist or antagonist co-activation from Pre- to Post-immobilisation. Previous research on EMG responses to immobilisation point to large decreases in EMG amplitude measurements during flexion in both the agonist and antagonist muscle (Vaughan, 1989; Yue et al., 1997). Vaughan et al. (1989) examined the EMG characteristics during extension and found significant decreases occurred in agonist peak EMG amplitude and antagonist peak EMG amplitude. Care should be taken when drawing conclusions from the EMG findings of the above as well as the present study. Indeed, a) the dimensional changes in the muscle could mean that a different population of motor units is likely being recorded from (Clark and Fielding, 2012); b) the reliability of EMG assessment in the present study (CV = ~11 % within day, and ~20 % between days) as in previously published studies

(de Araujo et al., 2009; Fukuda et al., 2010) is not very high, and this is a general limitation of studies utilising longitudinal EMG monitoring.

There was no significant changes in IL-6 from Pre- to Post-immobilisation in either EAA or PLA group. Research in humans demonstrates an increase in cytokine release in response to periods of bed-rest (Biolo et al., 2008; Bosutti et al., 2008; Hojbjerre et al., 2011). The muscle investigated in the current study is small (upper arm contains ~5.1 % of body mass) and as such any endocrine effects would be limited when compared to bed-rest in which more of the musculature is sedentary (Biolo et al., 2008; Bosutti et al., 2008; Hojbjerre et al., 2011). As mentioned in the introduction, there is a link between increased fatty tissue deposition (Manini et al., 2007), and increased expression and plasma levels of cytokines, such as IL-6 (Ahima and Flier, 2000; Pedersen et al., 2003). In the present study, it would appear that any observed changes in subcutaneous adipose tissue was not great enough to impact on circulating IL-6 levels.

Correlation analyses revealed that those participants who experienced a greater change in triceps brachii muscle thickness also had a greater change in triceps brachii sub-cutaneous adipose thickness. This suggests that any adaptations in the triceps brachii with immobilisation occur in both the muscle and the sub-cutaneous adipose content. Unsurprisingly, greater changes in upper arm girth were associated with greater changes in lower arm girth, denoting that both the directly immobilised, as well as anatomically proximate musculature, exhibit deleterious responses to increased local hypo-activity. Changes in upper arm girth were associated with elbow extension torque. The changes to the musculature, therefore, appear to be not only quantitative but also qualitative. Similarly, the fact that relative changes in IL-6 levels was associated with changes in upper arm girth and elbow extension torque, suggests that increased circulating IL-6 may only be a small reflection of greater changes at a cellular level.

2.5. Conclusion

A trend for a positive effect of EAA supplementation on upper and lower arm girth Post-immobilisation was observed. The multi-ingredient nature of the supplement makes it difficult to identify which specific amino acid or combination thereof, may have been responsible for the observed changes. Based on the literature, the likelihood is that leucine was the main effector (for a recent review, read Breen and Phillips (2012). It is suggested that the current results warrant future research in arm immobilisation of similar duration. In particular, it is proposed that the model used in this current study could have relevance to a sporting population in which short-term immobilisation may be prescribed (e.g. treatment for minor injury). The use of single sex populations, as well as multiple sites being monitored along the length of the muscle are proposed. Single sex populations are suggested, to rule out any potential differences in the response to immobilisation between males and females. In this manner, the overall impact of EAA supplementation (e.g. on elbow flexors/ extensors) may be further clarified.

Chapter 3: Omega-3 fatty acids and vitamin D in immobilisation: modulation of appendicular mass content, composition and structure

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3.1. Introduction

As discussed in Chapters 1 and 2 skeletal muscle undergoes structural adaptations in response to changes in functional demand. Prolonged periods of reduced muscle activity and mechanical loading, e.g. immobilisation, limb-suspension or bed-rest, result in changes in skeletal muscle structure and function, bone mineral density (BMD) and intermuscular adipose content (Bloomfield, 1997; de Boer, Maganaris, et al., 2007; LeBlanc et al., 1992; Manini et al., 2007; Rittweger et al., 2005; Veldhuizen et al., 1993). Musculoskeletal trauma and sports injuries are often treated with orthopaedic surgery/ limb immobilisation, which inevitably leads to periods of hypo-activity and disuse. The prognosis of orthopaedic surgery patients is poor especially in the older population with a ~6 to 17 % mortality rate within three years of hip and knee joint replacement (National Joint Registry, 2012). Exercise could be beneficial in these circumstances, but is not always practical. In addition, there is a notable need to identify non-pharmacological interventions since polypharmacy in itself is conducive to skeletal tissue loss (Moylan and Binder, 2007). Nutritional supplementation may serve as a valid intervention for attenuating the effects of disuse (summarised in Chapter 1). Chapter 2 investigated the potential of EAA and now the current Chapter introduces two further supplements.

Eicosapentaenoic acid (EPA) is an n-3 polyunsaturated fatty acid with anti-inflammatory properties, which is synthesised from ingested alpha-linolenic acid or consumed in fish, or in fish oil, such as sardines and cod liver oil. Despite there being no established Dietary Reference Intake for ω-3 fatty acids, adequate intake (AI) is set at 1.6 and 1.1 g/day for men and women, respectively (A Report of the Panel on Macronutrients et al., 2005). There is evidence to suggest that EPA may reduce the pro-inflammatory cytokines associated with muscle damage-induced

inflammation (Magee et al., 2008). Magee et al. (2008) demonstrated *in vitro* that EPA inhibits the effects of tumour necrosis factor alpha (TNF-α) by reducing its apoptotic effects and enabling myogenesis. It is unclear whether this supplement would have a beneficial effect during immobilisation, where it is generally accepted that there is muscle atrophy (Grosset and Onambele-Pearson, 2008), which is associated with decreased protein synthesis (de Boer, Selby, et al., 2007) but scant evidence for increased protein breakdown (Ferrando et al., 1996).

Another non-pharmacological agent that may potentially be used against the asthenia and atrophy induced through immobilisation is vitamin D. Vitamin D is required to absorb calcium and phosphorus and plays a crucial role in maintaining bone, muscle function, modulation of cell growth, neuromuscular and immune functions, and reduction of inflammation. The main source of vitamin D is sunlight, with smaller amounts found in certain foods. The Recommended Dietary Allowance for vitamin D is 600 IU/day (Ross et al., 2011). Vitamin D supplementation is another way of making sure the recommended allowance is achieved. Vitamin D has been shown to have direct effects on muscle (Ceglia and Harris, 2012); however, the exact mechanisms remain unknown. To date, research has indicated an association between genetic variation in the vitamin D receptor (VDR) gene and muscle strength, fat mass and body mass in premenopausal women (Grundberg et al., 2004). Moreover, vitamin D has been reported to impact on both the trans-membranous flows of calcium and phosphate in skeletal muscle, and the synthesis rate of contractile properties (Stewart and Rittweger, 2006). Vitamin D supplementation reduced falls by 49 % and improved musculoskeletal function in frail elderly women with vitamin D deficiency (Bischoff et al., 2003). It remains to be seen whether vitamin D supplementation in healthy persons with no known vitamin D deficiency, would lead to any enhancement in muscle structural and contractile properties in the presence of immobilisation.

In the present study, an arm immobilisation model was chosen as it is relatively less restrictive on daily life and causes fewer burdens on participants. The aim was to determine the role that the two potential protein-sparing modulators (EPA or vitamin D supplementation) may play in attenuating atrophy induced through a model that would emulate relatively short-term decreased local mobility/activity in humans. The differential effect of immobilisation on *in vivo* muscle (thickness), limb composition (lean mass, bone parameters and fat mass), and anthropometry (arm girth) was systematically monitored. Participants received either omega 3 (ω-3, a fish oil of a complex of EPA and docosahexaenoic acid (DHA)), vitamin D or placebo (Lecithin), hereafter simply referred to as EPA, vitamin D or placebo supplementation. It was hypothesised that muscle thickness, lean mass and arm girth will decrease with limb immobilisation. It was also hypothesised that EPA will be the most effective supplement at minimising these changes, since it is understood to act on the protein synthesis pathways.

3.2. Methods

3.2.1. Participants

Twenty-four healthy volunteers were recruited from the local university campus. All participants provided written informed consent before taking part in this study, which was approved by the local Ethics Committee of Manchester Metropolitan University. Exclusion criteria were any conditions requiring the use of medication likely to affect muscle function or musculoskeletal health (e.g. statins and oral steroids), and any current or history of kidney/liver disease, as those suffering with such conditions are more susceptible to the side effects of nutritional supplementation. A questionnaire (Appendix 4) to ascertain health and habitual physical exercise levels prior to the study confirmed all participants were

recreationally active and free from recent (last 6 months) upper limb injury or nutritional supplementation. Participants were randomly assigned to one of three groups by an independent person, using block randomisation: placebo [PLA: n = 8]; EPA [EPA: n = 8]; or vitamin D [Vit-D: n = 8]. For anthropometric measurements in the three populations, please see the results section (Table 3.1).

3.2.2. Study design

The study used a randomised, double-blind, placebo-controlled design. All participants attended a familiarisation session at least one week prior to the first testing session. All testing sessions were completed after an overnight fast. After baseline testing, the non-dominant arm was immobilised in a sling, with the correct sling wearing procedure demonstrated to each participant (Figure 2.1, Chapter 2). The non-dominant arm was chosen to minimise the burden on participants. Participants were required to wear the sling for nine waking hours a day for two continuous weeks, removal of the sling was permitted only when necessary (e.g. taking a bath/shower, driving, sleeping etc.), minimising any movement medio-laterally at the elbow and shoulder, whilst requiring participants to not contract the upper musculature (including the hands) during the hours of immobilisation. Measures of upper arm muscle and sub-cutaneous fat thickness, body composition (lean mass, bone parameters and fat mass), and upper and lower arm girth were taken immediately before immobilisation (Pre), on removal of the sling (Post), and two weeks after re-mobilisation (Post2).

The PLA group consumed two 1200 mg capsules of Soya Lecithin (Holland & Barrett, UK) a day, each daily dose typically providing 1464 mg of Phosphatides. The EPA group consumed three softgel of High EPA Formula (MorEPA, Minami Nutrition, Belgium), with the daily dose providing 1770 mg EPA and 390 mg DHA. The Vit-D group consumed one softgel of Vitamin D_3 (Now Foods, Bloomingdale,

U.S.A.), each dose providing 1,000 IU of Vitamin D_3. The participants consumed the nutritional supplements during the two weeks of limb immobilisation. Participants were asked to maintain their habitual diet and not to perform any unaccustomed strenuous exercise during the 2 weeks of immobilisation and remobilisation. To monitor this, during the immobilisation period participants completed a 3-day food diary, a daily activity log (including sling-wear hours) and wore a pedometer (Omron Walking style III step counter, Omron Healthcare Co., Ltd, Kyoto, Japan) to record the number of steps taken each day. The food diaries were analysed for macronutrient and micronutrient average intake using Microdiet Plus 1.2 (Microdiet, Downlee Systems Ltd, UK). All participants recorded their daily sling wear hours and were questioned during their final testing session, to confirm their compliance with the protocol.

3.2.3. Muscle and sub-cutaneous adipose thickness measures

All images were recorded after approximately 20 minutes seated rest to avoid fluid shifts that might induce interstitial and/or intracellular changes (Berg, Tedner, et al., 1993). Images of the muscle and sub-cutaneous adipose tissue of the upper arm were obtained using B-mode ultrasonography (AU5, Esaote, Genoa, Italy). A 7.5-MHz linear phased-array probe (image depth: 37.1-92.8mm) was applied in the sagittal plane with minimal pressure to the tissue area of interest to avoid image distortion. This method has previously been shown to be highly reliable for determining muscle and adipose thickness (Miyatani et al., 2004; Miyatani et al., 2002; Onambele et al., 2006). Images were recorded using Adobe Premiere 6.0 (Adobe Systems, USA) and stored for later analysis.

Images were obtained with the participant in an upright, seated position with their arm abducted square to the body (elbow at 0˚) and resting on the ultrasound machine. In the upper arm, the proximal and distal insertions of the biceps and

triceps brachii were identified by sonography and marked on the skin. The midpoint (L50) and a third of the distance (L33) from the distal end of the biceps and triceps brachii were identified and marked onto the skin (Figure 3.1). Upper arm ultrasonography images were collected in the sagittal plane, at both sites on both the biceps and triceps brachii. Muscle thickness was measured as the distance from the top of the superficial muscle aponeurosis to the bone at both sites along the biceps and triceps brachii. Ultrasound assessment of muscle tissue content has previously been validated (Giles et al., 2014; Dupont et al., 2001; Temes et al., 2014; Abe et al., 2014). Sub-cutaneous adipose thickness was measured as the distance from the bottom of the epidermis to the top of the superficial muscle aponeurosis in the biceps and triceps at both sites. Each of these distances were measured at three standardised points along the width of the probe (Figure 2.2, Chapter 2) to obtain average muscle and sub-cutaneous adipose thicknesses using ImageJ analysis software (ImageJ 1.37, Maryland, USA).

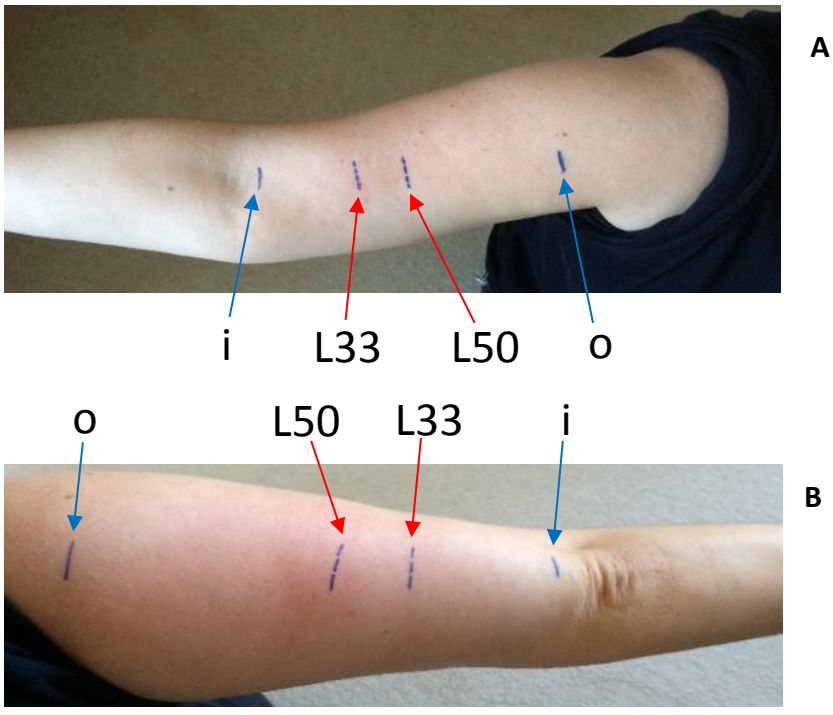

Figure 3.1. Insertion (i), origin (o), L50 and L33 markings on the biceps (A) and triceps (B).

3.2.4. Body composition analysis

Body composition was determined using dual-energy x-ray absorptiometry (DXA) scanner (Hologic Discovery; Vertec Scientific, Reading, Berkshire, UK). Whole body scans were performed (Figure 3.2) lasting approximately seven minutes with a dose-area product (DAP) of 21 cGy*cm^2. The appendicular mass was isolated from the trunk and head using DXA regional computer-generated default lines, with manual adjustment, on the anterior view planogram. Measures of BMD, bone mineral content (BMC), lean mass, fat mass and fat percentage are reported for the immobilised arm only.

Figure 3.2. DXA scan set-up.

3.2.5. Arm girths

Participants assumed a relaxed standing position with arms hanging by the sides and palms facing the hips. A measuring tape was used to measure upper arm girth at the mid-acromial-radial and lower arm girth at a fixed point a third of the way (from the proximal end) along the length of the radiale-stylion. Measurements were repeated three times at each point and average girths were calculated.

3.2.6. Measurement reliability

All protocols were assessed for intra as well as inter-day reliability. This utilised five participants and entailed carrying out measurements on two repeat visits. The two visits were conducted at the same time of day, approximately a week apart. Each ultrasound scan and arm girth measurement were taken three times on each day and analysed as per the method section. Within-day coefficient of variation (CV) of 0.1 %, 0.1 %, 0.4 %, 0.2 %, 0.3 % and 0.3 %, and between-day CVs of 0.4 %, 0.2 %, 0.6 %, 0.2 %, 0.2 % and 0.4 % were yielded for upper arm girth, lower arm girth, biceps muscle thickness, triceps muscle thickness, biceps subcutaneous adipose thickness and triceps subcutaneous adipose thickness, respectively. DXA reliability is reported with a CV of 1.0 %.

An investigation to assess the impact of small limb rotations on DXA-recorded bone parameters, used a bovine tibia bone, placed on a hinged Perspex device on the DXA bed (Figure 3.3). The hinged device allowed for controlled and quantifiable rotations of the bone model, through custom designed, laser cut Perspex triangles holding the device at specific angles (Figure 3.3) ranging from 0° to 50° (5° increments). A DXA forearm scan protocol (forearm length set at 42cm, scan length set at 15.3 cm) was performed twice at every angle. Each scan was analysed for bone area, BMC, BMD, T-scores and Z-scores. Intraclass correlation coefficient (ICC) was used to assess the consistency between repeated scans at each angle. ICC values of 0.95, 0.92, 0.98, 0.97 and 0.97 were yielded for bone area, BMC, BMD, T-scores and Z-scores, respectively. Bone area and BMC decreased with increasing angle rotation. BMD, T-scores and Z-scores increased with increasing angle rotation of the bovine tibia bone. The effect of bone rotation angle was assessed by linear regression. The regression coefficients R = 0.975 ($p<0.05$), 0.945 ($p<0.05$), 0.946 ($p<0.05$), 0.945 ($p<0.05$) and 0.943 ($p<0.05$) were yielded for bone area, BMC, BMD, T-scores and Z-scores, respectively.

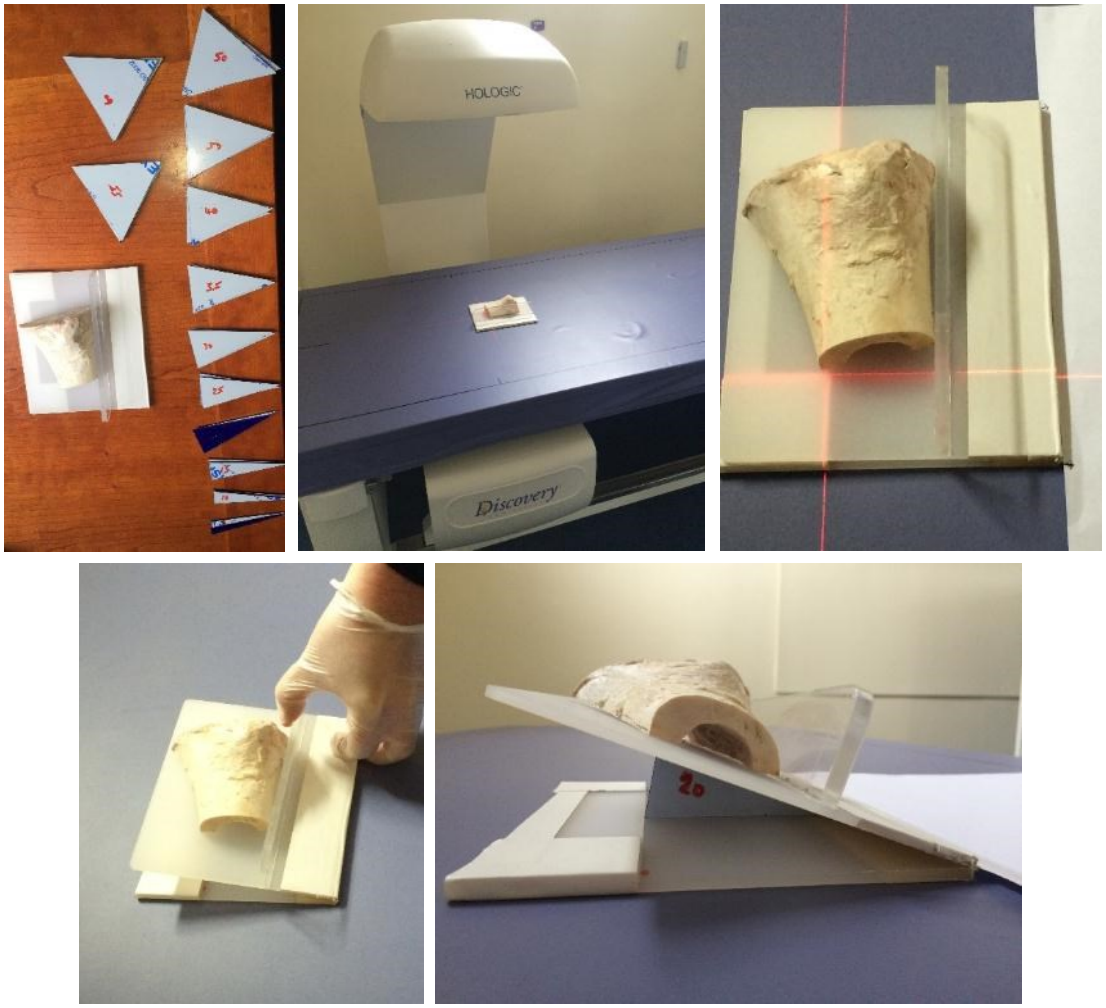

Figure 3.3. Set-up of the DXA for the assessment of limb rotation on DXA bone parameter outputs.

3.2.7. Statistical analyses

Data were analysed using IBM SPSS v21 (IBM Inc, USA). The Shapiro-Wilk test revealed some of the data to be non-parametric (upper arm muscle and adipose thickness, upper and lower arm girths). The effect of immobilisation was examined by assessing the changes seen in the PLA group by either repeated measures ANOVA (parametric data) or a Friedman test (non-parametric data). Parametric change relative to baseline values (Pre-to-Post: (Post-Pre)/Pre; and Pre-to-Post2: (Post2-Pre)/Pre) were analysed using a repeated measures ANOVA, with post-hoc Bonferonni corrected 2-tailed t-tests to determine group difference. Non-parametric between group effects on change data were analysed using the Kruskal Wallis test, with post-hoc Mann-Whitney U tests. All data are presented as mean ± standard deviation (SD). Statistical significance was set with alpha at ≤ 0.05.

3.3. Results

3.3.1. Homogeneity of sample

There were no significant differences in baseline characteristics between the groups (Table 3.1). The groups similarly did not differ in baseline muscle size (e.g. bicep muscle thickness (L50) - PLA: 28.7 ± 7.1 mm; EPA: 31.1 ± 5.4 mm; Vit-D: 32.1 ± 6.5 mm), arm girth (PLA: 28.8 ± 3.0 cm; EPA: 28.1 ± 4.0 cm; Vit-D: 29.4 ± 3.2 cm), nor body composition (e.g. lean mass – PLA: 1874.5 ± 595.8 g; EPA: 2361.0 ± 1116.8 g; Vit-D: 2531.1 ± 832.6 g).

Table 3.1. Baseline characteristics of all participants.

	PLA	EPA	Vit-D
Age (years)	26 ± 6.7	19 ± 1.6	23 ± 5.9
Males	n = 2	n = 4	n = 3
Females	n = 6	n = 4	n = 5
Height (cm)	168.7 ± 11.1	169.5 ± 12.0	172.2 ± 8.0
Mass (kg)	69.1 ± 14.2	69.6 ± 23.1	75.2 ± 14.5

3.3.2. Daily physical activity and nutritional intake

No significant differences were observed in calorific intake (PLA: 1797 ± 934 kcal/day; EPA: 1647 ± 551 kcal/day; Vit-D: 1960 ± 756 kcal/day ($p>0.05$)) or in habitual physical activity (PLA: 6825 ± 1660 steps/day; EPA: 7529 ± 3310 steps/day; Vit-D: 6145 ± 2081 steps/day ($p>0.05$)) between the groups during the course of the immobilisation. This effect was true for the EPA, Vit-D and PLA groups. Further diet composition analyses revealed no group differences in protein (PLA: 1.1 ± 0.3 $g \cdot kg^{-1} \cdot bw/day$; EPA: 1.0 ± 0.3 $g \cdot kg^{-1} \cdot bw/day$; Vit-D: 1.0 ± 0.6 $g \cdot kg^{-1} \cdot bw/day$), carbohydrate (PLA: 3.1 ± 1.5 $g \cdot kg^{-1} \cdot bw/day$; EPA: 3.1 ± 1.1 $g \cdot kg^{-1} \cdot bw/day$; Vit-D: 3.0 ± 1.0 $g \cdot kg^{-1} \cdot bw/day$), fat (PLA: 1.0 ± 0.5 $g \cdot kg^{-1} \cdot bw/day$; EPA: 1.0 ± 0.5 $g \cdot kg^{-1} \cdot bw/day$; Vit-D: 1.0 ± 0.3 $g \cdot kg^{-1} \cdot bw/day$), vitamin D (PLA: 1.7 ± 0.7 µg/day; EPA: 1.6 ± 0.9 µg/day; Vit-D: 2.0 ± 1.6 µg/day) or ω-3 (PLA: 0.37 ± 0.30 g/day; EPA: 0.36 ± 0.19 g/day; Vit-D: 0.36 ± 0.20 g/day) intake over the immobilisation period between the three groups.

Results of the questionnaire revealed that participants walked to and from work, university and/or shopping a minimum of 30 minutes per day. Examination of

the activity diaries completed by the participants themselves during the immobilisation period, confirmed that the majority of participants spent at least half an hour outside each day.

3.3.3. Muscle and sub-cutaneous adipose thickness measures

Muscle thickness decreased from Pre to Post at both sites (L50 and L33) on the biceps and triceps brachii ($p<0.05$) in the PLA group. The percentage decrease in muscle thickness was significantly greater in the triceps brachii (L50 & L33) than the biceps brachii (L33) ($p<0.05$) The percentage change in muscle thickness at both sites on the biceps and triceps brachii did not significantly differ between the groups (Figure 3.4), though both EPA and Vit-D groups showed a non-significant trend towards differing from the PLA group (bicep L50 $p=0.078$; bicep L33 $p=0.063$; triceps L50 $p=0.072$; triceps L33 $p=0.074$). Sub-cutaneous adipose thickness increased significantly Post immobilisation at L50 of the biceps brachii ($p<0.05$), with no significant change at L33 of the biceps brachii or either site on the triceps brachii. The percentage change in adipose thickness was not significantly different between groups at both sites on the triceps brachii (Figure 3.4). Percentage change in adipose thickness was significantly greater in the PLA than the EPA group (L50 $p=0.028$; L33 $p=0.015$) and in the Vit-D than the EPA group (L50 $p=0.038$; L33 $p=0.028$) at L50 and L33 along the biceps brachii (Figure 3.4).

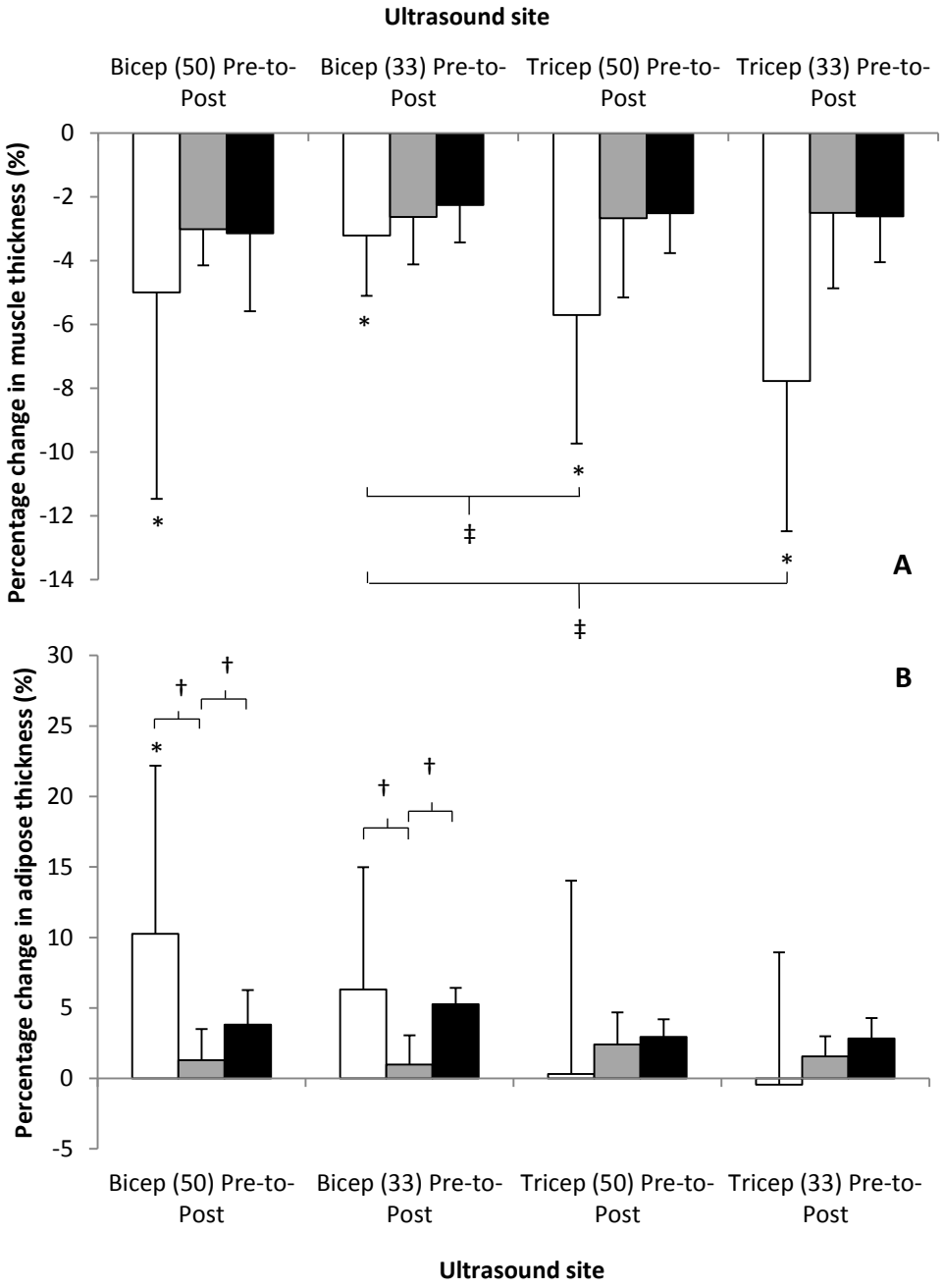

Figure 3.4. Percentage change (% ± SD) in muscle thickness (A) and sub-cutaneous adipose thickness (B) from Pre-to-Post for PLA (white bars), EPA (grey bars) and Vit-D (black bars) at the midpoint (L50) and a third of the distance (L33) along the length of the biceps and triceps brachii. * Significant difference between Pre and Post immobilisation in the PLA group. † Significant difference in % change between groups. ‡ Significant difference in % change values between ultrasound sites.

3.3.4. Body composition

There was a significant decrease in lean mass Post immobilisation ($p<0.03$) but no significant effect of supplement group on the percentage change in lean mass ($p=0.09$) (Figure 3.5). Percentage changes in BMC, BMD, fat mass and fat percentage are displayed in Table 3.2. No significant change was observed in BMD, fat mass or fat percentage. There was a significant decrease in BMC from Pre to Post immobilisation in the PLA group ($p<0.05$). There was no significant difference in percentage change in BMC, BMD, fat mass or fat percentage between the groups Post or Post2 ($p>0.05$).

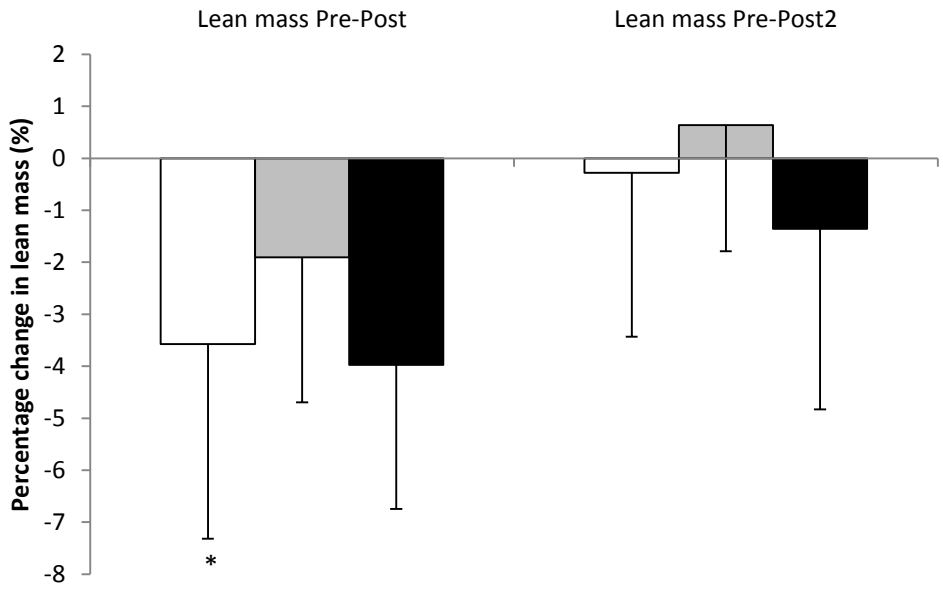

Figure 3.5. Percentage change (% ± SD) in lean mass from Pre-to-Post and Pre-to-Post2 for PLA (white bars), EPA (grey bars) and Vit-D (black bars). * Significant difference between Pre and Post immobilisation in the PLA group.

Table 3.2. Percent changes (% ± SD) in immobilised limb composition in response to immobilisation and supplementation. * Significant difference between Pre and Post immobilisation in the PLA group.

	PLA		EPA		Vit-D	
	Pre-to-Post	Pre-to-Post2	Pre-to-Post	Pre-to-Post2	Pre-to-Post	Pre-to-Post2
BMC	-2.3 ± 1.5 *	-1.5 ± 1.1	-0.3 ± 1.0	0.6 ± 1.9	-0.7 ± 1.9	-0.6 ± 0.7
BMD	-1.6 ± 2.6	0.4 ± 2.2	-0.5 ± 3.2	-0.3 ± 1.1	0.0 ± 1.5	0.5 ± 2.1
Fat mass	2.0 ± 2.4	-1.4 ± 6.7	3.2 ± 3.2	0.2 ± 1.7	2.0 ± 3.5	1.9 ± 1.8
Fat %	2.1 ± 5.9	0.0 ± 5.2	2.6 ± 5.3	-1.2 ± 4.3	3.4 ± 2.6	0.4 ± 3.6

3.3.5. Arm girths

Upper and lower arm girths significantly decreased from Pre to Post immobilisation ($p<0.05$) in the PLA group. There was no significant difference in the percentage change in upper or lower arm girth between groups (Figure 3.6).

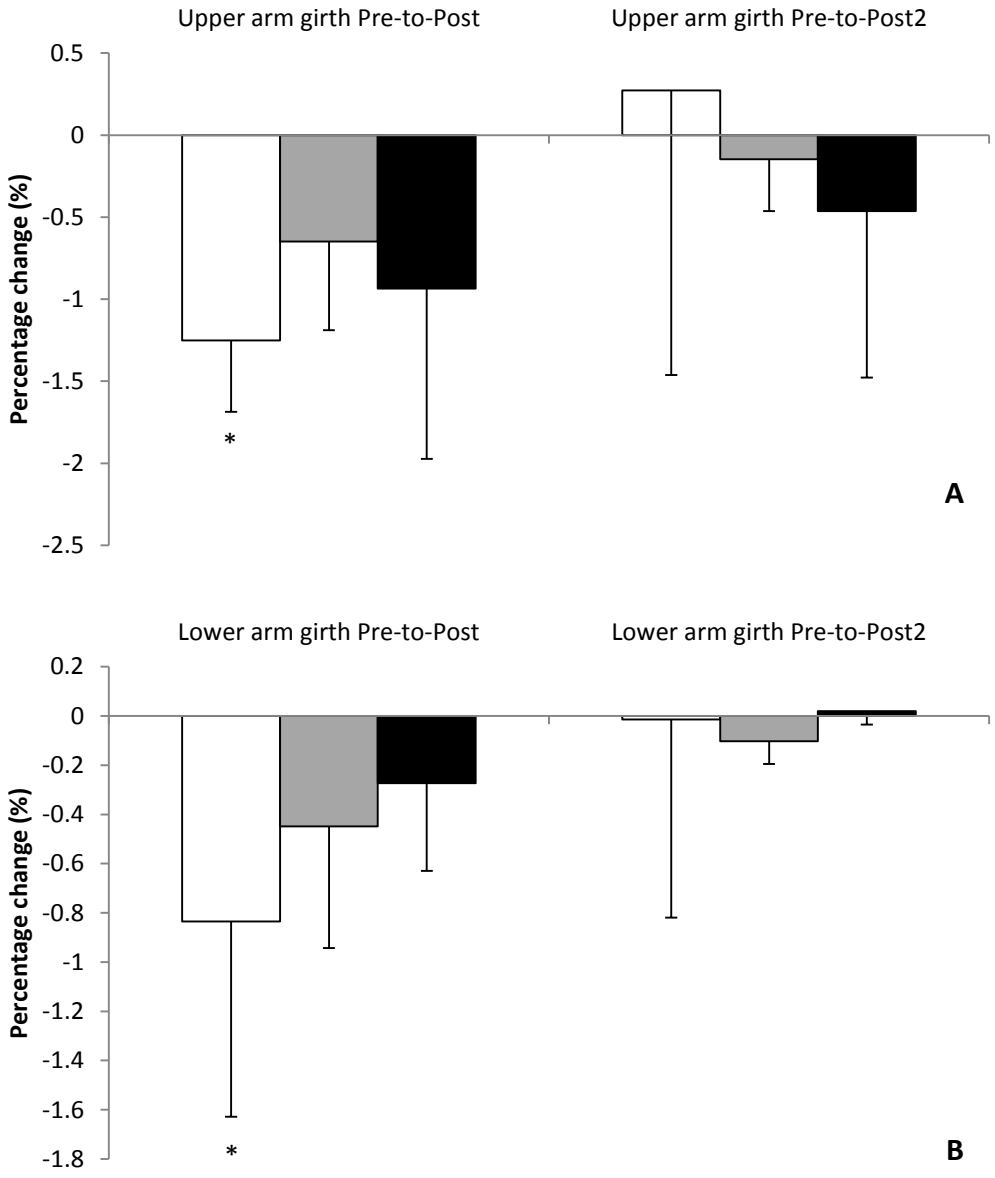

Figure 3.6. Percentage change (% ± SD) in upper (A) and lower (B) arm girth from Pre-to-Post and Pre-to-Post2 for PLA (white bars), EPA (grey bars) and Vit-D (black bars). * Significant difference between Pre and Post immobilisation in the PLA group.

3.3.6. Non-immobilised limb data

All parameters were also recorded on the non-immobilised, control limb. A phase specific degree of change was calculated to obtain one value for control limb corrected relative change for each variable (phase control-corrected change = immobilised limb percentage change – non-immobilised limb percentage change). The 'phase control-corrected change' values were analysed using the same statistical analysis process as those used for the immobilised limb only results. The outcome revealed that normalisation for the non-immobilised limb made no difference on any of the outcome parameters ($p>0.05$) apart from sub-cutaneous adipose thickness at the L33 site on the biceps ($p<0.05$). There was a significant decrease in muscle thickness, arm girth, lean mass and BMC and a significant increase in sub-cutaneous adipose thickness at both sites on the biceps brachii. As in the immobilised limb only data, the only significant effect of supplement group was on the percentage change in adipose thickness. This change was significantly greater in the PLA than the EPA group ($p<0.05$) and in the Vit-D than the EPA group ($p<0.05$) at L50 and L33 along the biceps brachii. Due to the lack of impact of normalisation for the non-immobilised limb, it was decided that only the immobilised limb data would be presented.

3.4. Discussion

The purpose of this study was to determine the role that two potential protein-sparing modulators (EPA or vitamin D supplementation) may play in attenuating the deleterious physiological changes induced through 2 weeks of 9-waking-hours-per-day combined arm and shoulder immobilisation. It was hypothesised that muscle thickness, lean mass and arm girth would decrease with limb immobilisation. Evidence was found to support this, with significant decreases in

muscle thickness (PLA: -3.2 to -7.8 % dependent on anatomical site), arm girth (PLA: -0.8 to -1.3 % dependent on anatomical site) and lean mass (PLA: -3.6 % for the immobilised limb). In addition, there was a significant increase in sub-cutaneous adipose thickness (PLA: 10.3 %) and a significant decrease in BMC (PLA: -2.3 %). It was also hypothesised that EPA would be the most effective supplement at minimising the effects of immobilisation. In fact, the data show that neither EPA nor vitamin D had any significant effect on the responses to non-injurious immobilisation, other than on the associated accumulation of sub-cutaneous fatty tissue. Nonetheless, a few trends ($p<0.1$) towards attenuations in deleterious physiological events were observed, in the EPA and Vit-D treated groups. The observed trends for the attenuation of some parameters are discussed.

A significant change in upper limb muscle thickness is evidenced in the ultrasound data, with a significant decrease in biceps and triceps brachii muscle thickness with immobilisation. The ultrasound data demonstrate a greater decrease in triceps brachii muscle (L50 and L33) thickness than the biceps brachii (L33) in the PLA group. It was expected, in fact, that the muscle held in the shortened position (i.e. the biceps) to be impacted on more than the muscle held in the lengthened position (i.e. the triceps); previous research suggests that sarcomeres are added in series when the muscle is immobilised in the lengthened position, and sarcomeres are lost when the muscle is immobilised in the shortened position (Williams and Goldspink, 1973). In the present study, the elbow was immobilised at a 90° angle and, as such, would not have exerted maximal lengthening or shortening on the triceps and biceps brachii. The EPA and Vit-D supplementation groups demonstrated non-significant trends towards smaller decreases in muscle thickness in response to immobilisation, suggesting that EPA and vitamin D may have some role to play in attenuating muscle atrophy

associated with hypo-activity. A recent study in an animal model demonstrated distinct effects of EPA and DHA on protein metabolism (protein synthesis and breakdown) with EPA showing a greater ability to result in skeletal muscle protein accretion. Further work in humans is required to further investigate this and the effects EPA may have in human disuse models. Indeed, it is likely that the dose, duration and/or whole body measurement of muscle mass that was adopted in these animal studies diminished the comparability of animal models from the immobilisation model used in this current study.

Upper and lower arm girths were shown to decrease significantly Post immobilisation in the PLA group. Arm girth was used as a gross marker of skeletal muscle atrophy, as previously used by Matsumura et al (2008). A decrease in upper arm girth (in the PLA group) suggests a decrease in muscle CSA in the upper arm and this supports the ultrasound data along with previous findings of a decrease in elbow flexor muscle CSA and volume with arm immobilisation (Yue et al., 1997). The decrease in lower arm girth suggests a decrease in forearm muscle CSA, and this is in line with findings from Miles et al. (1994) who reported a decrease in forearm muscle CSA with 9 days arm casting. The significant decrease in limb CSA is reflected in the DXA data, with a significant decrease in lean mass with immobilisation. The decrease in upper arm girth appeared to be attenuated by EPA and vitamin D supplementation, with EPA having the greater effect. Similarly, in the lower arm, supplementation showed a trend towards attenuating the losses in arm girth, this time with vitamin D having a slightly greater effect. The EPA group appear to show a smaller decrease in lean mass than both other groups.

The ultrasound data revealed no significant change in sub-cutaneous adipose thickness in the triceps brachii over the immobilisation period. Biceps sub-cutaneous adipose thickness, however, did significantly increase at the midpoint

site in the PLA group. Manini et al. (2007) reported a significant increase in intermuscular adipose tissue and no significant change in subcutaneous adipose tissue in response to 4-weeks unilateral lower limb suspension (ULLS). The difference between the response of sub-cutaneous adipose tissue to disuse in the current study in comparison to Manini et al. (2007) may be due to the different techniques used to assess the parameter (i.e. ultrasound vs. magnetic resonance imaging (MRI)) and/or the differing modes of immobilisation (i.e. arm immobilisation vs. ULLS). It is possible that the more stringent immobilisation in their study (throughout daily mobile activities, for four weeks), as well as the adiposity site (intermuscular), may account for the fact that they observed significant increases in adiposity. The present study partially agrees with their findings as the current data showed a significant increase at one region, subcutaneously, along the upper arm (i.e. 50 % of upper limb length). The EPA group demonstrated a significantly smaller increase in biceps sub-cutaneous adipose thickness following immobilisation than both the PLA and Vit-D groups. This effect may, indeed, be meaningful given that diet was monitored and the dietary records demonstrated neither time nor between group differences in total calories, macronutrients or vitamin D intakes, throughout the study. EPA supplementation shows potential for attenuating increases in sub-cutaneous adipose thickness in response to disuse. The Vit-D group also demonstrated a trend towards an attenuation in the increase of sub-cutaneous adipose thickness in comparison to the PLA group.

DXA analysis revealed no significant changes in fat mass or fat percentage with limb immobilisation. The difference in the response of adiposity to disuse in the current study in comparison to Manini et al. (2007), who reported a significant increase in intermuscular adipose tissue, may be due to the different techniques used to assess the parameter (i.e. DXA vs. MRI) or the different mode and/or

duration of disuse (i.e. 2 weeks arm immobilisation vs. 4 weeks ULLS). Interestingly, the increases reported in the present study in sub-cutaneous adipose thickness were not matched by the DXA analysis of fat mass or percentage. Possible explanations for this include: a) the DXA takes the average of the whole limb rather than regional data and hence would have 'missed' the regional changes observed with the ultrasound; b) it is possible that the tissue density changes with immobilisation mean that the DXA may have miss-qualified muscle and fat after immobilisation (Roubenoff et al., 1993).

There was no significant change in BMD (-0.8 %/week), but a significant decrease in BMC (-1.2 %/week) following immobilisation. Decreases in bone are often reported in more severe and longer periods of disuse. For example, Rittweger et al. (2005) reported significant decreases in BMC of the tibia (-0.1 to -0.5 %/week) and radius (-0.03 to -0.05 %/week) in response to 90 days bed-rest. Marchetti et al. (1996) found significant decreases in BMD (-1.0 to 2.3 %/week) in response to 6 weeks arm immobilisation, however, the participants had also undergone surgery, which could contribute to greater decreases in BMD. Values for BMC and BMD changes with immobilisation showed a trend towards attenuation of changes with EPA and vitamin D supplementation. Previous research indicates that BMD is affected by changes in body weight and composition (Van Loan et al., 1998), therefore, the observed changes in muscle and sub-cutaneous adipose thickness may have influenced the BMD values. The value of BMD can also be limited by the inherent limitations of using a two-dimensional x-ray projection to estimate bone area and geometrical changes. The use of peripheral quantitative computed tomography (pQCT) may have been more advantageous to examine these specific changes.

The formula of each supplement was chosen so that the dose provided would be able to be bought over the counter and be taken without prescription.

The chosen dose of EPA and DHA of the ω-3 supplement in the current chapter is in line with previous levels used in the literature (Bloomer et al., 2009; Lenn et al., 2002; Phillips et al., 2003). The vitamin D dose of 1000 IU is equal to 25 µg. Public Health England suggest that adults at risk of vitamin D deficiency should take a daily supplement containing 10 µg of vitamin D (Public Health England, 2014). The selected dose is much higher than this and as such should be sufficient in the healthy population used in the current chapter. The lack of the effectiveness of vitamin D and EPA in the current study may be due to the dosage of the supplements used and therefore this needs to be altered, potentially increased, in future studies.

The NHS consensus on vitamin D states that if people achieve a sufficient supply of vitamin D in the summer, most should keep levels greater than the deficiency threshold of 25nmol/l in winter even without supplements (NHS Livewell, 2010). In the current study, participants reported that they typically spent at least 30 minutes outdoors every day of the week and as such would reach the necessary sunlight exposure for adequate vitamin D level. We therefore suggest that the current population are unlikely to be vitamin D deficient. The 'healthy, presumably non-deficient' status of the participants may have been another reason for the lack of effectiveness of the supplementation in the current study.

Blinding of ω-3 supplementation is sometimes an issue in studies, due to the fact that it often repeats on participants. On completion of the study, participants were asked whether they were able to identify what supplement they believed they were taking. None of the participants correctly guessed the supplement they were taking and in fact, two participants receiving the placebo supplement believed they were taking an ω-3 supplement.

3.5. Conclusion

In accordance with previous research, the results of this study demonstrate a decrease in muscle thickness, arm girth and lean mass with upper limb immobilisation. A significant decrease in BMC was also observed, with no effects of immobilisation on fat mass or BMD. Interestingly nonetheless, it was observed that in the case of sub-cutaneous adiposity on the biceps brachii, there is a protective effect of EPA supplementation against limb immobilisation. It is proposed that the model used in the current study could have relevance to a sporting population in which short-term immobilisation may be prescribed (e.g. treatment for minor injury) or in clinical populations (e.g. injury/surgery induced short-term immobilisation/bed-rest).

Chapter 4: Omega-3 fatty acids and vitamin D in immobilisation: modulation of muscle functional, vascular and electromyographic profiles

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4.1. Introduction

Chapter's 1 to 3 show that skeletal muscle adapts to environmental changes and differing levels of physical activity. It has consistently been demonstrated, that disuse models, including immobilisation, bed-rest and limb-suspension result in skeletal muscle atrophy (Berg et al., 1997; Clark et al., 2006; Miles et al., 1994), a decrease in maximal voluntary strength (Berg et al., 1997; Clark et al., 2006; Miles et al., 1994), and changes in electromyographic characteristics (Vaughan, 1989). Numerous studies have also documented the effects of immobilisation models on muscle fatigability, with equivocal findings of both decreased, increased and no change in resistance to fatigue (Miles et al., 1994; Miles et al., 2005; Yue et al., 1997). With progressing fatigue, there is a shift in electromyography (EMG) to lower frequencies, and median power frequency can be used as an index of this frequency shift. A decrease in the median power frequency serves as an index of fatigue (Knaflitz et al., 1990; Soderberg and Knutson, 2000). Fast Fourier transform (FFT) of EMG produces a discrete-time, discrete-frequency representation and can be used to determine median power frequency. There are also several reports of the impact of disuse on the cardiovascular system with reports of decreased reactive hyperaemic blood flow (Gayeski and Honig, 1983; Henriksson and Reitman, 1977; Shoemaker et al., 1998). Early research demonstrated that static muscular contractions were accompanied by a marked impairment in blood flow to exercising muscles (Barcroft and Millen, 1939). Since then impaired blood flow has been used as an explanation for muscle fatigue during isometric contractions. Local changes in vascular dimensions and blood flow characteristics have been shown in response to resistance training, with de-training resulting in a worsening in these parameters (beyond pre-training values)

(Stebbings et al., 2013). Therefore, decreased loading with disuse, may negatively impact on vascular structure and function.

The ever-increasing population of frail elderly puts a strain on healthcare services, with increased disability and functional impairment, cognitive decline and the accumulating burden of disease due to the obesity epidemic. Indeed, prolonged sedentarism/hypo-activity as may be encountered in enforced bed rest, immobilisation owing to orthopaedic clinic events, or simply even, decreased habitual physical activity, all show a high incidence in older persons (D'Antona et al., 2003; Sayer et al., 2008; Morley et al., 2001; Marcell, 2003; Dock, 1944). A common and serious problem for older adults is falls, with polypharmacy and some medications contributing to falls in many patients (Moylan and Binder, 2007). This factor however, is remediable and non-pharmacological interventions are needed to prevent the age-associated loss in muscle size and function. As discussed in the previous chapters, exercise could be beneficial in these circumstances but is not always a practical prescription; and as such, nutritional interventions could be key.

As described in Chapter 3 (i.e. the previous study), an arm immobilisation model was chosen as it is relatively less restraining on daily life and causes less burden on participants. The potential of omega 3 (ω-3) and vitamin D as nutritional supplements, to attenuate disuse atrophy are discussed and investigated in Chapter's 1 and 3. In Chapter 3, whilst there was no significant effect of supplement group on muscle size decreases with immobilisation, a non-significant trend for lesser atrophy in the treatment groups was seen. Based on the greater decline in muscle strength (due to the combined effects of neural and muscle components) with disuse, it is possible that functional measures are more sensitive to immobilisation than structural changes. It is possible therefore, that

EPA and vitamin D may preserve muscle function to a greater extent than a placebo over the period of immobilisation.

The aim, therefore, was to determine the role that vitamin D or EPA supplementation may play in attenuating the changes associated with limb immobilisation. The differential effect of immobilisation on isometric and isokinetic elbow torque, agonist co-contraction, muscle fatigability and resting arterial blood flow (vessel diameter, heart rate, resistance index and flow by diameter), was systematically monitored with participants receiving either ω-3 (a fish oil of a complex of EPA and docosahexaenoic acid (DHA)), vitamin D or placebo (Lecithin), hereafter simply referred to as EPA, vitamin D or placebo supplementation. It was hypothesised that muscle function will decrease, muscle co-contraction characteristics will change, and indices of healthy vascular function will deteriorate, with limb immobilisation. It was also hypothesised that EPA would be the most successful supplement at minimising these changes, as it acts on the protein synthesis pathways.

4.2. Methods

4.2.1. Participants & study design

Participant inclusion criteria and study design were as described in study Chapter 3. Briefly, twenty-four healthy volunteers participated in the study, following appropriate ethical approval, and then randomly assigned to one of three groups (placebo (PLA): n = 8 (6 females, 2 males); EPA: n = 8 (4 females, 4 males); vitamin D (Vit-D): n = 8 (5 females, 3 males)). The study used a randomised, double-blind, placebo-controlled design with the placebo group consuming 1464 mg Soya Lecithin (Holland & Barrett, UK) daily, the Vit-D group consuming 1,000 IU of Vitamin D_3 (Now Foods Bloomingdale, U.S.A.) daily, and the EPA group

consuming 1770 mg EPA plus 390 mg DHA (MorEPA, Minami Nutrition, Belgium), daily during the immobilisation period.

Participants attended a familiarisation session at least one week prior to the first testing session. After baseline testing, the non-dominant arm was immobilised in a sling for a minimum of nine waking hours a day, for two continuous weeks. The correct sling wearing procedure was demonstrated to each participant (Figure 2.1. of Chapter 2), the removal of the sling was only permitted when necessary (e.g. driving, taking a bath/shower etc.). The sling minimised any movement medio-laterally at the elbow and shoulder and participants were required to not contract the upper musculature (including the hands) during immobilisation hours. Measures of isometric and isokinetic elbow torque, EMG co-contraction, muscle fatigability and arterial dimensions and blood flow, were taken immediately before the immobilisation period (Pre), immediately after the immobilisation period (Post), and two weeks after remobilisation (Post2). During the immobilisation period, participants completed a 3-day food diary, a daily activity log and wore a pedometer to record the number of steps taken each day. The techniques and equipment used are described in Chapter 3. Nutritional information and steps taken each day are reported in the results section of Chapter 3.

4.2.2. Dynamometry

Isometric and isokinetic elbow torque were assessed using a Cybex dynamometer (Cybex, New York, USA). Participants were positioned as per the manufacturer's recommendations. Briefly, they were positioned in a supine position with the axis of rotation of the dynamometer aligned with the anatomical axis of rotation of the elbow joint (lateral epicondyle) (Figure 4.1).

Figure 4.1. Participant positioning and Cybex dynamometer set-up.

4.2.3. Isometric dynamometry

Following a warm up at 60°/sec at the participant's self-perceived ~75 % of maximum effort, two repetitions of isometric contractions were performed at six different elbow joint angles (60°, 70°, 80°, 90°, 100° and 110°), 60 seconds apart. The highest torque was recorded as the participant's maximal voluntary contraction (MVC) for each angle. Participants were instructed to rapidly exert maximal torque against the dynamometer lever arm over a 3-4 second period. First in flexion and, five seconds after return to baseline, in extension. Torque and angle were displayed on the screen of a computer (Macintosh G4; Apple Computer, Cupertino, CA), which was interfaced with an A/D system (Acknowledge, Biopac Systems, Santa Barbara, CA), with a sample frequency of 200 Hz. Participants were encouraged to exert maximal torque with the use of visual and verbal feedback. Peak torque was averaged over a 500 ms period (i.e. 250 ms either side of the instantaneous peak). The highest of the repeated efforts was used as the participant's measure of MVC at each angle for elbow flexion and extension.

4.2.4. Isokinetic dynamometry

Isokinetic contractions were completed with three continuous elbow extensions and flexions at six different speeds (30, 60, 90, 120, 180 and 240°/sec) separated by 90 seconds, in a randomised order. The highest of the three consecutive efforts was recorded as peak torque (25 ms either side of the instantaneous peak) for elbow extension and flexion at each speed.

4.2.5. Electromyographic measurements

Muscle activation patterns were assessed using EMG during the isometric and isokinetic contractions The skin was prepared by shaving, abrading and cleaning with an alcohol-wipe to minimise resistance below 5 kΩ (Hermens et al., 2000). Self-adhesive electrodes were placed in pairs either side of the marker of a third of the distance along the biceps and triceps brachii, with reference electrodes placed on the lateral and medial epicondyle of the humerus. Raw EMG data were recorded at 2000 Hz, with a band pass filter set at 10-500 Hz, and a notch set at 50 Hz (Biopac Systems). Biceps co-contraction was calculated (biceps EMG during extension / biceps EMG during flexion) for both isometric and isokinetic contractions. Triceps co-contraction was also calculated (triceps EMG during flexion / triceps EMG during extension) again for both isometric and isokinetic contractions. Biceps and triceps EMG values were taken during the same windows as in isometric and isokinetic torque.

4.2.6. Fatiguing contractions

The dynamometer lever arm was locked at a 90° angle (where 0° is full elbow extension) and participants were required to exert a maximal isometric contraction in the direction of elbow flexion for 30 seconds. After 90 seconds of recovery, the participant then repeated the maximal isometric contraction for 30 seconds, this

time in the direction of elbow extension. The mean, slope and standard deviation of the torque trace were recorded for the length of the 30-second contractions. FFT was computed for the agonist muscle during the first five and last five seconds of each fatiguing contraction. Median frequency values were determined for these time points and a change in median frequency was then computed as a measure of the fatigability of the muscle.

4.2.7. Arterial resting blood flow

After more than 20 minutes seated rest (following muscle ultrasound scans), allowing for the regulation of vascular tone, measurements in the sagittal plane of resting brachial artery diameter, heart rate (HR), resistance index (RI) and flow by diameter (FbD) were obtained from the ultrasound software (Figure 4.2). The measurements were obtained using an echo Doppler ultrasound machine (AU5, Esaote, Genoa, Italy) with a 5.0- 13.0 MHz broadband linear array transducer (with settings of Doppler gain 37-41, angle of insonnation 60 degrees). The ultrasound probe was applied to the arm in line with the marker of the midpoint of the biceps brachii (as previously marked earlier for obtaining muscle ultrasound images (Figure 3.1, Chapter 3). An average of nine data points were acquired for all measurements, with the mean value reported.

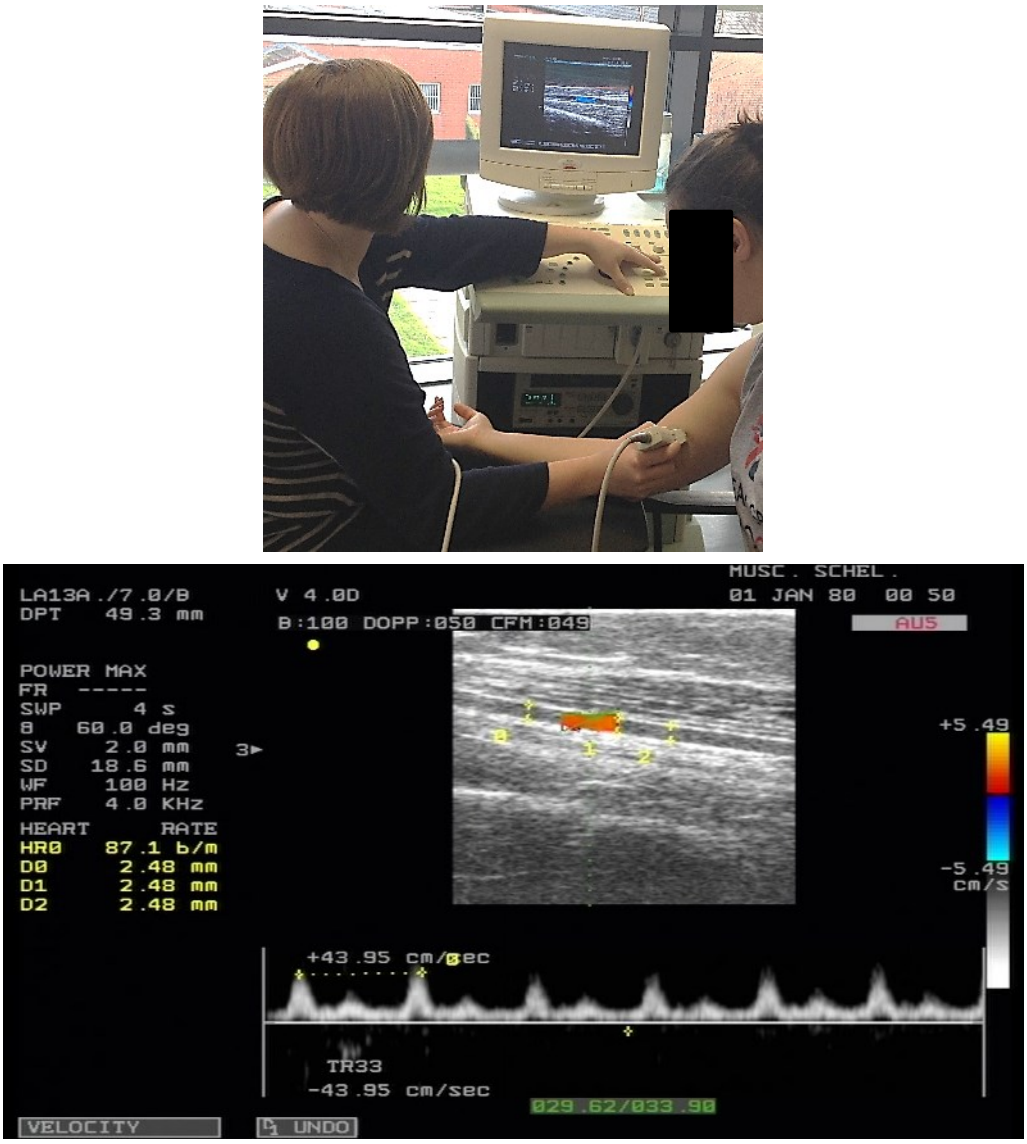

Figure 4.2. Ultrasound set-up and screen grab of arterial resting blood flow.

4.2.8. Measurement reliability

All protocols were assessed for intra as well as inter-day reliability. This utilised five participants and entailed carrying out measurements on two repeat visits. The two visits were conducted at the same time of the day, approximately a week apart. Isometric and isokinetic contractions were performed three times (at every angle and speed, in the direction of flexion and extension) by each participant on both visits. Arterial resting blood flow ultrasound scans were also taken three times on each visit. All measurements were analysed as per the method section; and within- and between-day coefficient of variation (CV) values calculated. Within-day CVs of 1.8 %, 2.0 %, 1.5 %, 5.4 %, 2.4 % and 12.0 %, and between-day CVs of 2.5 %, 2.3 %, 2.3 %, 8.1 %, 4.0 % and 9.1 % were yielded for isometric torque, isokinetic torque, brachial artery diameter, HR, RI and FbD, respectively.

4.2.9. Statistics

Data were analysed using IBM SPSS v21 (IBM Inc, USA). The Shapiro-Wilk test revealed some of the data to be non-parametric (EMG, fatigue, brachial artery diameter, HR and FbD values). The effect of immobilisation was examined by assessing the changes seen in the PLA group by either repeated measures ANOVA (parametric data) or a Friedman test (non-parametric data). Parametric percentage change values (Pre-to-Post: (Post-Pre)/Pre; and Pre-to-Post2: (Post2-Pre)/Pre) were analysed using a repeated measures ANOVA, with post-hoc Bonferonni corrected 2-tailed t-tests to determine group difference. Non-parametric between group effects were analysed using the Kruskal Wallis test, with post-hoc Mann-Whitney U tests. All data are presented as mean ± standard deviation (SD). Statistical significance was set with alpha at ≤ 0.05.

4.3. Results

4.3.1. Baseline characteristics

There were no significant differences in baseline characteristics (Table 3.1. of Chapter 3). Additionally, the groups did not differ in baseline isometric MVC elbow torque (e.g. isometric elbow torque at 90° for flexion - PLA: 37.6 ± 15.0 Nm; EPA: 42.0 ± 15.7 Nm; Vit-D: 42.3 ± 13.7 Nm) EMG and fatigue values, or resting arterial blood vessel characteristics (vessel diameter - PLA: 3.3 ± 0.5 mm; EPA: 3.3 ± 0.6 mm; Vit-D: 3.2 ± 0.3 mm, HR – PLA: 69.6 ± 11.6 bpm; EPA: 68.5 ± 12.1 bpm; Vit-D: 69.1 ± 6.7 bpm, RI – PLA: 0.7 ± 0.3; EPA: 0.8 ± 0.1; Vit-D: 0.9 ± 0.1, FbD – PLA: 0.11 ± 0.05 m/s; EPA: 0.07 ± 0.02 m/s; Vit-D: 0.06 ± 0.03 m/s).

4.3.2. Isometric dynamometry

Isometric MVC torque decreased for both elbow flexion (60° p=0.030; 70° p=0.005; 80° p=0.012; 90° p=0.019; 100° p=0.030) and extension (60° p=0.002; 70° p=0.017; 80° p=0.023; 90° p=0.005) at every angle except for flexion at 110° (p=0.586) and extension at 100° (p=0.904) and 110° (p=0.300). Average isometric torque decrease from Pre to Post immobilisation across angles for flexion were 12.1 ± 1.8 %, 11.4 ± 3.5 % and 8.1 ± 3.4 %, and for extension were 15.4 ± 3.3 %, 12.0 ± 3.1 % and 10.7 ± 2.4 %, for PLA, EPA and Vit-D, respectively. There was no effect of group on the percentage change in isometric torque for flexion or extension at any of the six angles (p>0.05) (Figure 4.3).

4.3.3. Isokinetic dynamometry

Isokinetic torque decreased significantly for both elbow flexion (30°/sec p=0.018; 60°/sec p=0.014; 120°/sec p=0.023; 180°/sec p=0.043; 240°/sec p=0.030) and extension (30°/sec p=0.003; 60°/sec p=0.009; 90°/sec p=0.003; 120°/sec p=0.005;

180°/sec p=0.014; 240°/sec p=0.013) at every speed except for flexion at 90°/sec (p=0.078). Average isokinetic torque decrease from Pre to Post immobilisation across speeds for flexion were 14.0 ± 4.5 %, 8.6 ± 1.3 % and 7.8 ± 5.5 %, and for extension were 10.3 ± 1.8 %, 8.1 ± 0.9 % and 7.1 ± 1.8 %, for PLA, EPA and Vit-D, respectively. There was no effect of supplement group on the percentage change in isokinetic torque for flexion or extension at any of the six speeds (p>0.05) (Figure 4.4).

4.3.4. Electromyographic measurements

Analysis of biceps and triceps co-contraction RMS EMG values during the isometric contractions showed no significant changes from Pre to Post immobilisation (p>0.05) and no effect of supplement group from Pre-to-Post and Pre-to-Post2 (p>0.05) (Figure 4.5). Similarly, this was the case for the isokinetic contractions with no significant effect of immobilisation (p>0.05) nor of supplement group (p>0.05) on the EMG response to the immobilisation (Figure 4.6).

4.3.5. Fatiguing contractions

The mean, slope and standard deviation of the torque traces showed no significant changes with immobilisation phase for either flexion or extension fatiguing contractions, with no effect of supplement group on percentage changes (p>0.05). Analysis of the FFT of EMG traces of the biceps and triceps brachii revealed no significant effect of immobilisation or supplementation group on rate coding at either the beginning, or the end of a fatiguing maximal isometric contraction (p>0.05) (Table 4.1).

4.3.6. Arterial resting blood flow

Brachial artery diameter, RI, FbD and HR did not change from Pre to Post immobilisation ($p>0.05$), nor Pre to Post2 ($p>0.05$). There was no significant difference in the percentage change in brachial artery diameter, RI, FbD or HR between the groups ($p>0.05$) (Table 4.2).

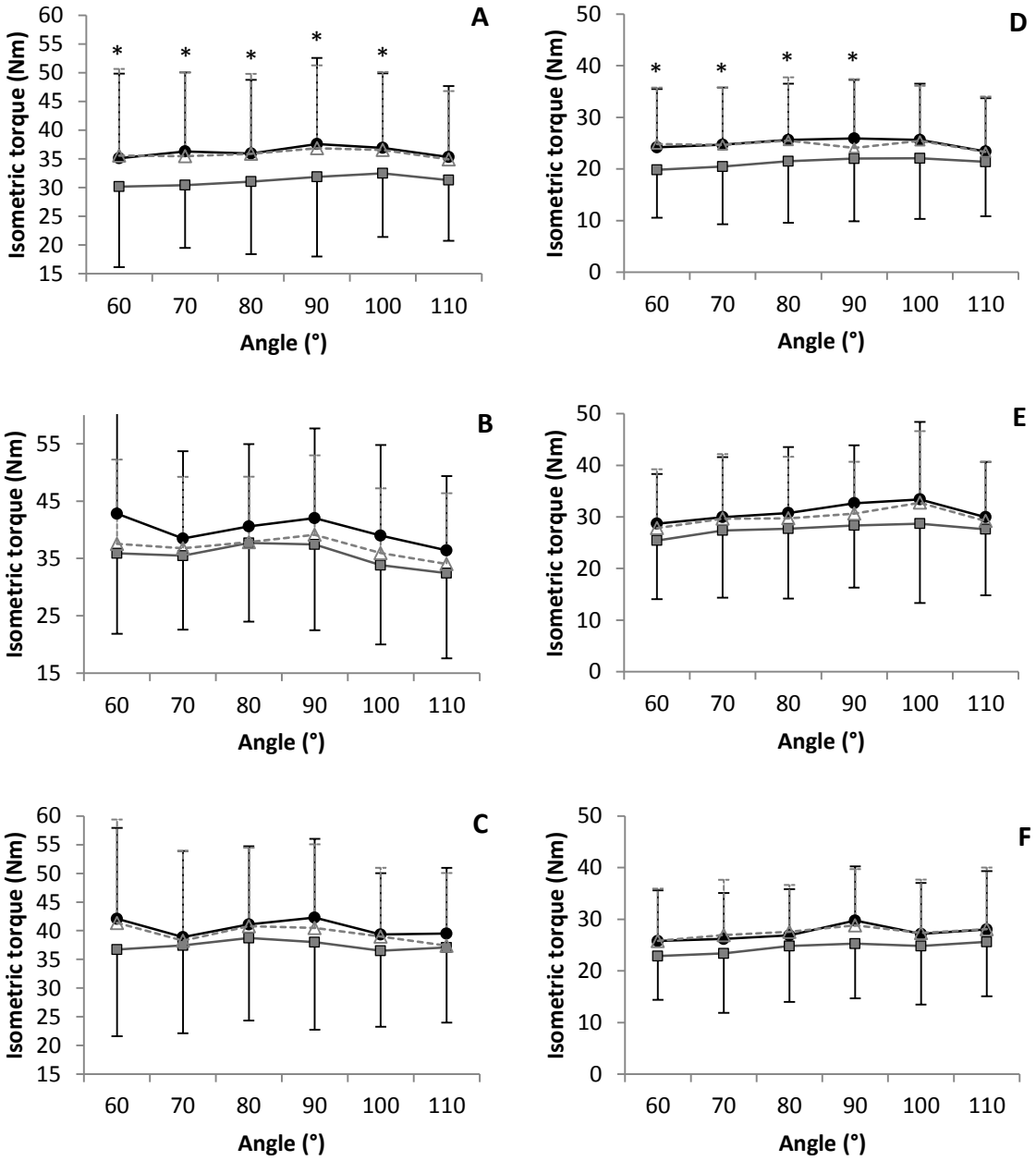

Figure 4.3. Pre (circles), Post (squares) and Post2 (triangles) values for isometric torque (Nm ± SD) for elbow flexion (A = PLA; B = EPA; C = Vit-D) and elbow extension (D = PLA; E = EPA; F = Vit-D) at the six different angles (60-110°). * Significant difference between Pre and Post immobilisation in the PLA group.

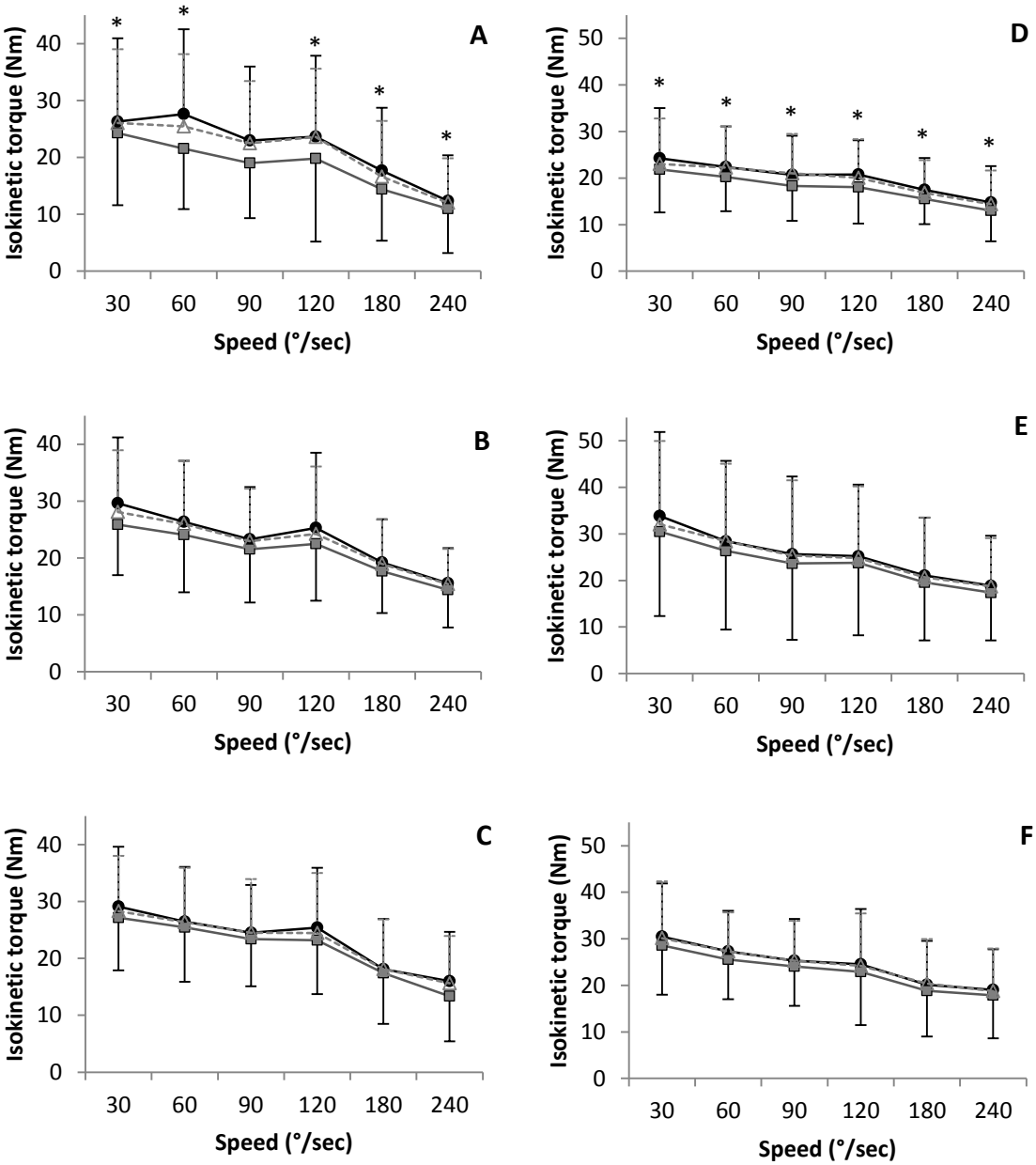

Figure 4.4. Pre (circles), Post (squares) and Post2 (triangles) values for isokinetic torque (Nm ± SD) for elbow flexion (A = PLA; B = EPA; C = Vit-D) and elbow extension (D = PLA; E = EPA; F = Vit-D) at the six different speeds (30-240°/sec). * Significant difference between Pre and Post immobilisation in the PLA group.

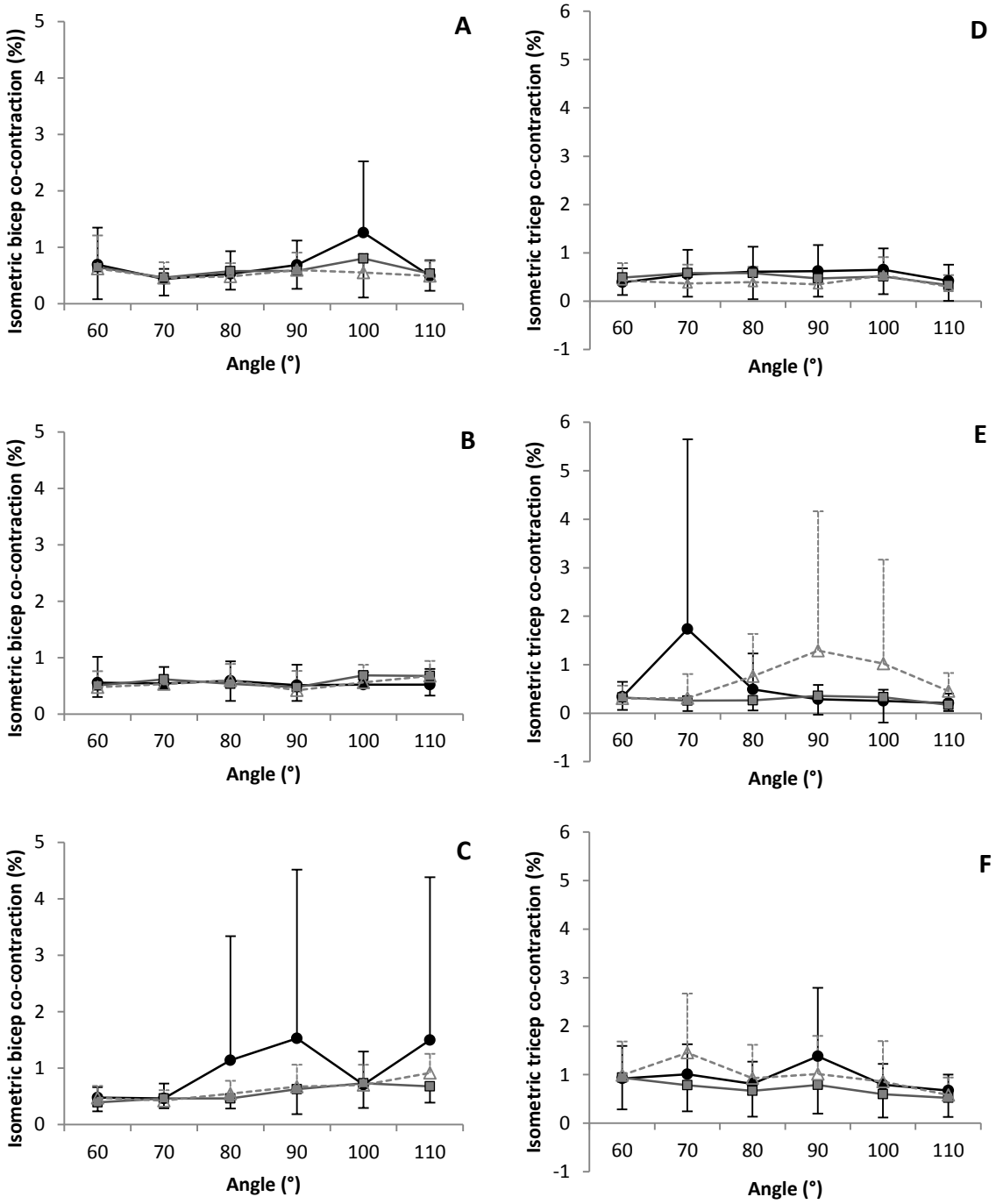

Figure 4.5. Pre (circles), Post (squares) and Post2 (triangles) values for co-contraction (Nm ± SD) of the biceps (A = PLA; B = EPA; C = Vit-D) and triceps (D = PLA; E = EPA; F = Vit-D) during isometric elbow flexion and extension at the six different angles (60-110°).

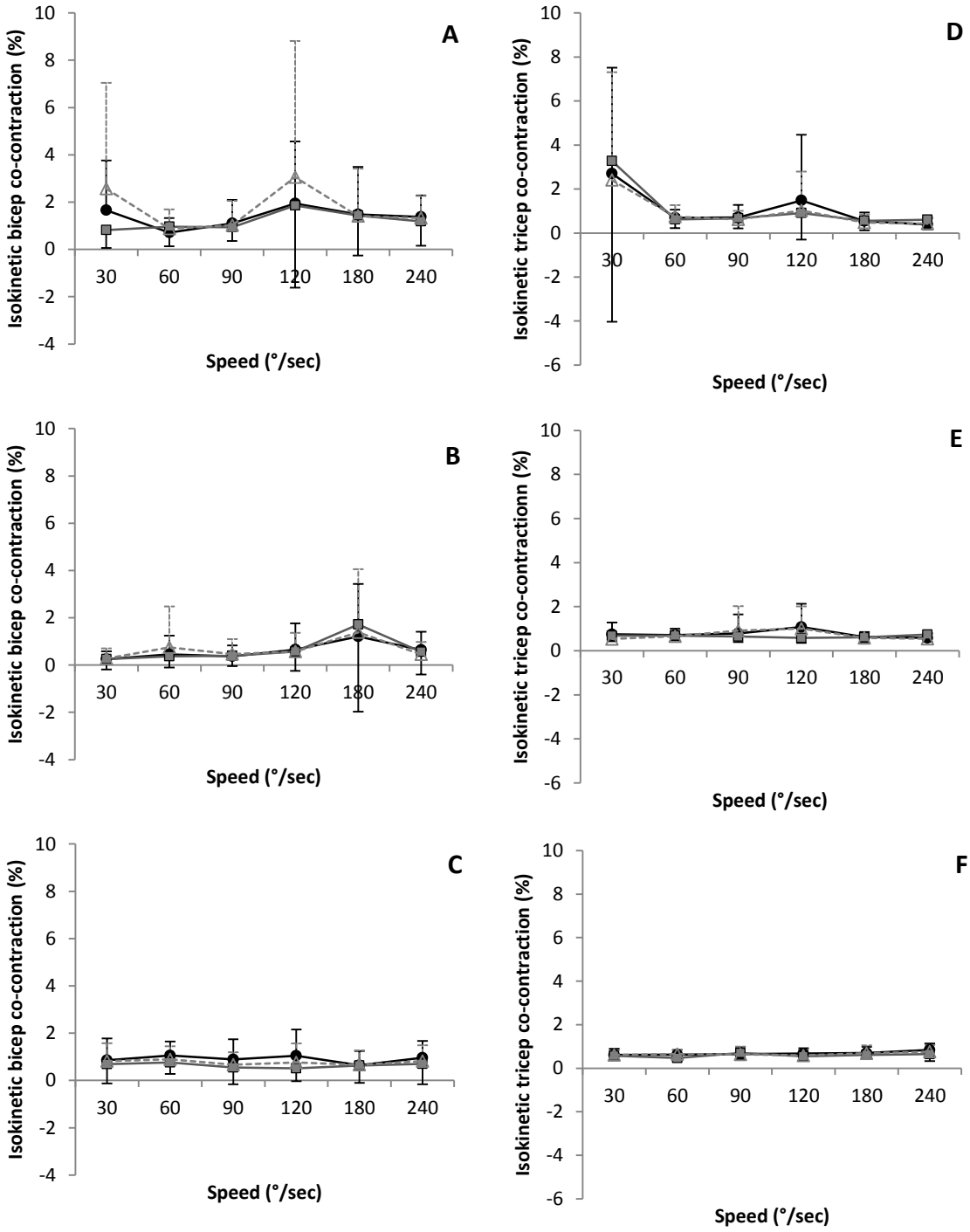

Figure 4.6. Pre (circles), Post (squares) and Post2 (triangles) values for co-contraction (Nm ± SD) of the biceps (A = PLA; B = EPA; C = Vit-D) and triceps (D = PLA; E = EPA; F = Vit-D) during isokinetic elbow flexion and extension at the six different speeds (30-240°/sec).

Table 4.1. Fatigue characteristics during a 30-second fatiguing contraction in flexion and extension in response to immobilisation and supplementation.

		PLA	EPA	Vit-D
Flexion mean torque (Nm)	Pre	26.2 ± 12.7	27.1 ± 11.3	28.4 ± 10.8
	Post	24.5 ± 12.1	23.6 ± 10.0	26.8 ± 10.9
	Post2	25.8 ± 12.1	24.7 ± 8.9	26.8 ± 11.4
Flexion standard deviation	Pre	3.2 ± 1.4	3.4 ± 1.3	3.0 ± 1.3
	Post	2.7 ± 0.8	2.9 ± 1.4	3.5 ± 1.7
	Post2	3.3 ± 1.3	2.7 ± 1.3	3.5 ± 1.7
Flexion Slope	Pre	-0.3 ± 0.2	-0.2 ± 0.1	-0.2 ± 0.1
	Post	-0.3 ± 0.2	-0.2 ± 0.1	-0.3 ± 0.2
	Post2	-0.3 ± 0.2	-0.1 ± 0.1	-0.1 ± 0.1
Flexion agonist FFT 1st five seconds (Hz)	Pre	113.6 ± 22.6	102.9 ± 8.5	102.3 ± 5.3
	Post	116.5 ±13.5	106.5 ± 7.8	103.9 ± 5.1
	Post2	112.1 ± 17.3	103.7 ± 8.0	102.1 ± 4.7
Flexion agonist FFT last five seconds (Hz)	Pre	115.2 ± 16.7	102.1 ± 8.0	104.2 ± 9.5
	Post	119.1 ± 15.8	105.5 ± 5.3	105.6 ± 8.5
	Post2	113.6 ± 12.7	101.8 ± 8.3	104.2 ± 9.2
Extension mean torque (Nm)	Pre	20.4 ± 14.4	23.8 ± 11.8	21.9 ± 12.1
	Post	19.2 ± 13.5	19.2 ± 8.7	17.7 ± 10.2
	Post2	21.3 ± 12.1	22.5 ± 10.7	22.3 ± 9.6
Extension standard deviation	Pre	2.9 ± 1.9	2.4 ± 1.6	2.2 ± 1.3
	Post	2.5 ± 1.5	2.5 ± 1.4	2.1 ± 0.9
	Post2	2.8 ± 2.1	2.2 ± 1.4	2.1 ± 1.1
Extension Slope	Pre	-0.3 ± 0.6	0.1 ± 0.1	-0.2 ± 0.3
	Post	-0.2 ± 0.2	-0.1 ± 0.2	-0.2 ± 0.2
	Post2	-0.3 ± 0.4	-0.1 ± 0.3	-0.1 ± 0.1
Extension agonist FFT 1st five seconds (Hz)	Pre	131.8 ± 13.6	128.9 ± 13.3	131.5 ± 9.7
	Post	132.6 ± 16.5	130.3 ± 12.0	133.2 ± 13.2
	Post2	142.3 ± 15.8	130.1 ± 14.4	131.7 ± 9.9
Extension agonist FFT last five seconds (Hz)	Pre	124.7 ± 17.0	124.1 ± 16.0	127.7 ± 12.4
	Post	125.0 ± 19.3	125.7 ± 15.3	129.4 ± 13.7
	Post2	124.3 ± 18.6	125.1 ± 15.9	128.5 ± 12.4

Table 4.2. Percent changes (% ± SD) in blood kinetic parameters in response to immobilisation and supplementation.

	PLA		EPA		Vit-D	
	Pre-to-Post	Pre-to-Post2	Pre-to-Post	Pre-to-Post2	Pre-to-Post	Pre-to-Post2
Brachial artery diameter	1.0 ± 14.7	3.7 ± 10.4	2.0 ± 10.8	2.4 ± 9.4	0.7 ± 8.2	3.6 ± 6.5
Resistance index (RI)	-4.6 ± 30.6	1.2 ± 20.7	-5.2 ± 15.1	-8.3 ± 9.1	-0.9 ± 15.1	-0.9 ± 15.1
Flow by diameter (FbD)	-14.6 ± 31.4	-8.8 ± 50.6	-7.3 ± 8.7	-12.9 ± 9.0	-9.5 ± 34.5	29.5 ± 15.5
Heart rate (HR)	-3.5 ± 11.0	-2.4 ± 6.9	4.0 ± 12.1	6.1 ± 15.7	4.3 ± 16.3	2.3 ± 14.9

4.3.7. Non-immobilised limb data

All parameters were also recorded on the non-immobilised, control limb. A phase specific degree of change calculation was completed to obtain one value for control limb corrected relative change for each variable (phase control-corrected change = immobilised limb percentage change – non-immobilised limb percentage change). The 'phase control-corrected change' values were analysed using the same statistical analysis process as those used for the immobilised limb only results. This revealed that normalisation for the non-immobilised limb made no difference on any of the outcome parameters. Due to the lack of impact of normalisation for the non-immobilised limb, it was decided that only the immobilised limb data would be presented.

4.4. Discussion

The effects of two potential protein-sparing modulators (EPA of vitamin D supplementations) on the response to 2 weeks of 9-waking-hours-per-day combined arm and shoulder immobilisation are described. It was hypothesised that muscle function will decrease, muscle co-contraction characteristics will change, and indices of healthy vascular function will deteriorate, with limb immobilisation. Evidence was found to partially support the hypotheses, with significant immobilisation-induced decreases in isometric elbow flexion (PLA: 6.7 to 18.4 %) and extension (PLA: 8.7 to 13.8 %) torque, as well as isokinetic elbow flexion (PLA: 9.3 to 13.7 %) and extension (PLA: 9.8 to 18.1 %) torque. It was also hypothesised that EPA would be the most effective supplement at minimising the response to immobilisation. The current data demonstrate that neither EPA nor vitamin D had any significant effect on the responses to non-injurious immobilisation. Nonetheless, a few trends towards (observation of $p<0.1$ when

analysing the effect of supplementation) the attenuation of elbow isometric and isokinetic torque immobilisation-induced decreases were observed, in the EPA and Vit-D treated groups. The observed trends for the attenuation of these parameters are discussed. It is also notable that this immobilisation model had no impact on the assessed co-contraction and muscle fatigability, or on the assessed blood flow kinetics.

Isometric elbow extensor and flexor torque decreased following immobilisation. This supports previous findings of decreases in isometric MVC of the elbow flexors in response to 4 weeks of elbow cast immobilisation (Yue et al., 1997). In addition to the established decline in isometric torque, disuse models also result in reductions in dynamic torque outputs. Cast immobilisation of the arm (9 days) also results in decreased concentric and eccentric strength for flexion and extension of the wrist (Miles et al., 1994). The current data also show a decrease in isokinetic strength for both elbow flexion and extension. However, muscle function in terms of isometric and/or isokinetic torque, did not show a significant effect of either EPA or vitamin D supplementation at the current doses, in spite of somewhat blunting the effect of immobilisation, as this protective effect was not statistically significant.

Data collected for agonist and antagonist EMG activity highlighted no differences in biceps or triceps co-contraction following immobilisation. In contrast, some of the previous research shows a large decrease in EMG amplitude measurements during flexion in both the agonist and antagonist muscle (Vaughan, 1989; Yue et al., 1997). When drawing conclusions from EMG findings care should be taken, as: 1) the changes in muscle dimensions could result in a different population of motor units being recorded from (Clark and Fielding, 2012); 2) EMG reliability in previously published studies is not very high, and this is a general limitation of studies utilising longitudinal EMG monitoring (de Araujo et al., 2009;

Fukuda et al., 2010). In addition, it is notable that EMG data in our, and previous work (Hermens et al., 2000), does not normalise the data for the clarity of the signal. Specifically, in Chapter 3 it was demonstrated that sub-cutaneous adiposity changed with immobilisation, hence the electromyographic signature would have differed (Solomonow et al., 1994).

Numerous studies have documented the effects of immobilisation models on muscle fatigability, with equivocal findings of both decreased, increased and no change in resistance to fatigue (Miles et al., 1994; Miles et al., 2005; Yue et al., 1997). In the current study, there were no significant changes in the mean, slope or standard deviation of the torque trace during the 30-second isometric contraction fatigue tasks that were implemented. Differences between studies could be due to the mode/duration of disuse or in the method used to test fatigue resistance since fibre recruitment level and pattern would vary with changes in contractile profile (Bogdanis, 2012). The mechanisms behind the varying effects of immobilisation on muscle fatigability are yet to be fully explained. The observation of no significant effect of immobilisation on the FFT of EMG traces at either the beginning or end of a maximal isometric contraction suggests no effect on motor unit rate coding and none on fibre type recruitment.

Previous research suggests that decreases in physical activity leads to detrimental vascular adaptations (Delp et al., 2000; Louisy et al., 1997). Bed-rest studies measuring leg blood flow report inconsistent results, with some showing no changes in leg blood flow and others demonstrating a decrease in leg blood flow after periods of bed-rest (10-41 days) (Convertino, Doerr andStein, 1989; Louisy et al., 1997; Pawelczyk et al., 2001; Takenaka et al., 1994). None of these bed-rest studies report data on changes in arterial vessel diameter. Studies investigating vascular changes in response to upper limb immobilisation are lacking. No significant dimensional changes in vasculature with upper limb immobilisation are

reported in the current study. Similarly, there was no observation of any significant changes in resting HR in response to immobilisation. Similarly, the assessment of resting arterial blood flow (RI and FbD) revealed no significant changes in response to immobilisation; however, the results reported in the current study were resting and not reactive blood flow and as such it would be less likely for any effect to be observed (Shoemaker et al., 1998). The lack of change in muscle fatigability, however, goes hand in hand with the absence of vascularisation-related alterations.

4.5. Conclusion

In summary, upper limb immobilisation resulted in a decrease in elbow isometric and isokinetic torque, with no observed effect on co-contraction, muscle fatigability or resting blood flow. No significant effect of EPA or vitamin D supplementation on any of these parameters were observed. Despite greater relative decreases in torque than in tissue composition (Chapter 3), there is no significant effect of EPA or vitamin D supplementation on the decreases in torque. It would appear that muscle function might be a less reliable marker of the effectiveness of a supplement against the impact of immobilisation than tissue composition.

Chapter 5: Systemic endocrine profile following a small muscle, short duration, limb immobilisation

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5.1. Introduction

Prolonged periods of disuse (e.g. limb immobilisation and head-down-tilt bed-rest) result in skeletal muscle atrophy (Abe et al., 1997; Akima et al., 2000) and declines in maximal voluntary strength (Clark et al., 2008; Hortobagyi et al., 2000; Miles et al., 1994; Semmler et al., 2000; Veldhuizen et al., 1993; Yue et al., 1997) (Refer to Chapter 1 for a review). Deconditioning of skeletal muscle is mainly characterised by a loss of muscle mass (Akima et al., 2001), decreased fibre cross-sectional area (Hortobagyi et al., 2000), reduced force (Miles et al., 1994), increased insulin resistance (Hirose et al., 2000) and transitions in fibre types (Trappe et al., 2004). The disuse-associated atrophy has been attributed to fundamental molecular mechanisms such as decreased protein synthesis, increased protein degradation, and suppression of bioenergetics pathways associated with mitochondrial function, and increased oxidative stress (Jankala et al., 1997; Kandarian and Jackman, 2006). Disuse-induced muscle atrophy is a highly ordered process that is controlled by interactions between intracellular signalling pathways.

A growing body of literature (Reid and Li, 2001; Hopkins, 1996; Wilcox et al., 1996; Wilcox et al., 1992; Wilcox et al., 1994) indicates that cytokines are involved in the regulation of skeletal muscle function. Resting healthy human muscles express cytokines in a fibre type specific manner, suggesting that cytokines may play specific regulatory roles in normal physiology (Plomgaard et al., 2005). The fibre type changes seen within disuse models, could therefore, be demonstrated through changes in circulating cytokines. In this study, three cytokines associated with inflammation were selected, including interleukin 6 (IL-6), interleukin 10 (IL-10) and tumour necrosis factor alpha (TNF-α). Along with the cytokines, creatine kinase (CK) and insulin-like growth factor 1 (IGF-1) were also

selected. CK was chosen as a marker of muscle damage. Raised levels of serum CK are closely associated with cell damage, muscle cell disruption, or disease, and these cellular disturbances can cause CK to leak from cells into blood serum (Totsuka et al., 2002). IGF-1 was selected, as it has been associated with muscle hypertrophy and atrophy (Barton et al., 2010; Barton-Davis et al., 1998; Schakman et al., 2005).

Exercise prescription is not always practical during periods of hypo-activity and, as such, other interventions are needed to attenuate or prevent the disuse-induced declines in muscle morphology and function (Chapters 3 and 4). Nutritional interventions are needed since polypharmacy in itself is conducive to skeletal tissue loss (Moylan and Binder, 2007). Two potential protein-sparing modulators omega 3 (ω-3, a fish oil of a complex of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) and vitamin D, hereafter simply referred to as EPA or vitamin D supplementation, may be nutritional interventions with potential for attenuating disuse-induced atrophy.

EPA is an n-3 polyunsaturated fatty acid with anti-inflammatory properties. There is *in vitro* evidence to suggest that EPA may reduce the pro-inflammatory cytokines associated with inflammation (Magee et al., 2008). It has been demonstrated *in vitro* that EPA inhibits the effects of TNF-α by reducing its apoptotic effects and enabling myogenesis (Magee et al., 2008). It is generally accepted that there is human muscle atrophy where limb immobilisation is enforced (Grosset and Onambele-Pearson, 2008) (Chapter 3), which is associated with decreased protein synthesis (de Boer, Selby, et al., 2007) and no change in protein breakdown in human models (Ferrando et al., 1996). Whether EPA supplementation would have a beneficial effect during immobilisation, therefore, remains to be seen.

Vitamin D plays a crucial role in maintaining bone, muscle function, modulation of cell growth, neuromuscular and immune functions, and reduction of inflammation. The vitamin D receptor (VDR) has been identified in a large number of human tissues (Reichel et al., 1989), indicating the potential for widespread effects. Vitamin D's biologically active metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) binding with VDR influences the expression of genes involved in cell development, growth and differentiation (Bikle, 2010; Walters, 1992). Vitamin D mediates calcium and phosphorus metabolism and has been shown to have direct effects on muscle (Ceglia and Harris, 2012); although, the exact mechanisms remain unknown. Vitamin D has been reported to impact on the synthesis rate of contractile proteins (Stewart and Rittweger, 2006). Vitamin D supplementation reduced falls by 49 % and improved musculoskeletal function in frail elderly women with vitamin D deficiency (Bischoff et al., 2003). It remains to be seen, whether vitamin D supplementation in healthy persons with no known vitamin D deficiency, would lead to any preservation of muscle structural and contractile properties in the presence of immobilisation.

Chapters 3 and 4 show significant atrophy (muscle thickness (ultrasound), lean mass (dual-energy x-ray absorptiometry) and arm girth) and declines in muscle strength in response to two weeks of arm immobilisation. This atrophy could be because of a change in endocrine factors. Whilst in the previous chapters supplementation did not significantly prevent or reduce skeletal muscle atrophy, but non-significant trends of attenuated atrophy they caused, could potentially be attributed to the early start of modulation of an endocrine cascade of changes.

The cellular and molecular events controlling the atrophic process have still not fully been explored, and further investigations to allow novel interventions to combat disuse-induced atrophy need to be implemented. The aim of the present study was, therefore, dual: 1) monitor the response of circulating CK activity,

cytokines and IGF-1 levels to immobilisation; 2) investigate any protective influence of EPA or vitamin D supplementation, on the anticipated immobilisation-induced deleterious changes in endocrine profile.

5.2. Methods

5.2.1. Participants

Twenty-one healthy volunteers were recruited from the local university campus (a sub-sample of the study population from Chapters 3 and 4). Briefly, a questionnaire (Appendix 4) to ascertain health and habitual physical exercise levels prior to the study confirmed all participants were recreationally active and free from recent (last 6 months) upper limb injury. Exclusion criteria were any conditions requiring the use of medication likely to affect muscle function or musculoskeletal health (e.g. statins and oral steroids), and any current or history of kidney/liver disease, as those suffering with such conditions are more susceptible to side effects of nutritional supplementation. The screening process ruled out any participants with neuro-musculoskeletal disorders that could affect muscle function. Ethical approval was obtained through the Department of Exercise and Sport Science, Manchester Metropolitan University and all participants signed informed consent prior to taking part in the study.

5.2.2. Intervention

Full details are described in Chapters 3 and 4. Briefly, the study used a randomised, double blind, placebo-controlled design. Participants were randomly assigned to one of three groups: placebo [PLA: n = 7]; EPA [EPA: n = 7]; or vitamin D [Vit-D: n = 7] (Table 5.1). Participants attended the laboratory on three occasions for venous blood samples to be taken. Samples were immediately before immobilisation (Pre), on removal of the sling (Post), and two weeks after re-

mobilisation (Post2). All testing sessions were completed after an overnight fast. After baseline testing, the non-dominant arm was immobilised in a sling, with the correct sling wearing procedure demonstrated to each participant (Figure 2.1, Chapter 2). Participants were required to wear the sling for nine waking hours a day for two continuous weeks, removal of the sling was permitted only when necessary (e.g. taking a bath/showering, driving, sleeping etc.), minimising any movement medio-laterally at the elbow and shoulder, whilst requiring participants to not contract the upper musculature (including the hands) during the hours of immobilisation.

Supplementation was taken during the immobilisation period. The PLA group consumed a daily dose of Soya Lecithin typically providing 1464 mg of Phosphatides (Holland & Barrett, UK) a day, each. The EPA group consumed a daily dose providing 1770 mg EPA and 390 mg DHA (MorEPA®, Minami Nutrition, Belgium). The Vit-D group consumed a daily dose providing 1,000 IU of Vitamin D_3 (Now Foods, Bloomingdale, U.S.A.). To monitor daily activity and nutritional intake participants completed a daily activity log (including steps taken and sling-wear hours) and a 3-day food diary (as described in detail in Chapters 3 and 4).

Table 5.1. Baseline characteristics of all participants.

	PLA	EPA	Vit-D
Age (years)	25.6 ± 7.0	19.3 ± 1.7	23.3 ± 6.0
Males	n = 2	n = 4	n = 3
Females	n = 5	n = 3	n = 4
Height (cm)	170.3 ± 11.0	170.1 ± 12.9	173.1 ± 8.2
Mass (kg)	70.9 ± 14.2	69.9 ± 25.0	72.5 ± 13.4

5.2.3. Circulating CK, cytokines and IGF-1

After a twelve hour, overnight fasting period a hospital-trained phlebotomist took a blood sample from the antecubital vein of the forearm, using a 21mL gauge needle (Terume, Neolus 100, Leuven, Belgium). The 5-10mL of venous blood was left to clot on crushed ice for up to 1 hour before being centrifuged (Thermo Scientific, IEC CL31 - CL31R Multispeed, Thermo Electron Corporation) at 4000 rpm at 0°C for 10 minutes to separate the serum from the blood cells. Using a 200-1000 µl pipette (Pipetman, Gilson®, Middleton, USA), the resulting serum sample was separated into three aliquots (~500 µl each) and stored in 1.5 mL microcentrifuge tubes (FlipTube®, Hampshire, UK) at -20°C until later analysis for CK, IL-6, IL-10, TNF-α and IGF-1. The sensitivity and intra-assay variability of the analysis kits are shown in Table 5.2.

CK levels were measured using a standard colorimetry procedure, measuring at optical density 340 nm (BioTek ELx800 96 well Microplate Reader) and immediately analysed (Gen5, version 2.0). Individual samples were run in duplicate using an EnzyChromTM CK Assay Kit (BioAssay Systems, Hayward, CA), reading enzyme activity after 20 minutes (25 minutes if necessary, with readings > 300 U/L at 20 minutes) and 40 minutes of exposure to the assay mix. An average of two to four readings were taken per sample, until the coefficient of variation (CV) of the repeated values was <10 %.

IL-6, IL-10, IGF-1 and TNF-α were individually analysed using the standard Quantikine enzyme-linked immunosorbent assay (ELISA) technique, measuring at optical density 450 nm (BioTek ELx800 96 well Microplate Reader) and immediately analysed (within 30 minutes) (Gen5, version 2.0). Individual samples were run in duplicate using ELISA kits for each cytokine (R&D Systems Inc. Minneapolis, USA).

Table 5.2. Manufacturer's assessed sensitivity and intra-assay variability of the colorimetry and ELISA analysis kits.

	Sensitivity	Intra-assay variability (i.e. Coefficient of variation)
CK	5 U/L	≤ 5.0 %
IL-6	< 0.7 pg/mL	2.6 %
IL-10	< 3.9 pg/mL	3.7 %
TNF-α	0.5-5.5 pg/mL	4.7 %
IGF-1	0.007-0.056 ng/mL	4.0 %

5.2.4. Statistical analysis

Data were analysed using IBM SPSS v21 (IBM Inc, USA). The Shapiro-Wilk test revealed the data to be non-parametric. The effect of immobilisation was examined by assessing the changes seen in the PLA group by Friedman tests (3 repeated measures within one group). Non-parametric comparisons of between group differences in endocrine changes relative to baseline values (Pre-to-Post: (Post-Pre)/Pre; and Pre-to-Post2: (Post2-Pre)/Pre) were analysed using the Kruskal Wallis tests, with post-hoc Mann-Whitney U tests. One-way analysis of variance (ANOVA) were used to determine any differences between groups in daily physical activity and nutritional intake during immobilisation. The change relative to baseline values, are displayed as boxplots in the figures, allowing for the distribution of the values to be seen. Statistical significance was set with alpha at ≤ 0.05.

A joint results and discussion section follows, allowing the comparison and discussion of each variable within the context of the literature.

5.3. Results and Discussion

5.3.1. Daily physical activity and nutritional intake

No significant differences were observed in calorific intake (PLA: 1949 ± 895 kcal/day; EPA: 1668 ± 592 kcal/day; Vit-D: 1924 ± 810 kcal/day ($p>0.05$)) or in habitual physical activity (PLA: 6970 ± 1737 steps/day; EPA: 7397 ± 3552 steps/day; Vit-D: 6724 ± 1389 steps/day ($p>0.05$)) between the groups during the course of the two-week immobilisation. This effect was true for the EPA, Vit-D and PLA groups. Further diet composition analyses revealed no group differences in protein (PLA: 1.1 ± 0.3 $g \cdot kg^{-1} \cdot bw/day$; EPA: 1.0 ± 0.3 $g \cdot kg^{-1} \cdot bw/day$; Vit-D: 1.0 ± 0.6 $g \cdot kg^{-1} \cdot bw/day$), carbohydrate (PLA: 3.4 ± 1.4 $g \cdot kg^{-1} \cdot bw/day$; EPA: 3.2 ± 1.2 $g \cdot kg^{-1} \cdot bw/day$; Vit-D: 3.0 ± 1.1 $g \cdot kg^{-1} \cdot bw/day$), fat (PLA: 1.1 ± 0.5 $g \cdot kg^{-1} \cdot bw/day$; EPA: 1.0 ± 0.5 $g \cdot kg^{-1} \cdot bw/day$; Vit-D: 1.0 ± 0.3 $g \cdot kg^{-1} \cdot bw/day$) or micronutrient intake over the immobilisation period between the three groups. Micronutrient intake values can be found in Table 5.3.

Table 5.3. Average daily micronutrient intake of all participants.

	PLA	EPA	Vit-D
Retinol (µg)	267.5 ± 141.1	198.6 ± 105.6	244.4 ± 187.7
Carotene (µg)	1737.0 ± 1245.9	1101.4 ± 1211.0	1802.9 ± 1432.5
Vitamin B6 (mg)	1.9 ± 0.8	1.5 ± 0.5	1.9 ± 1.2
Vitamin B12 (µg)	3.8 ± 1.4	3.1 ± 1.0	3.8 ± 3.0
Vitamin C (mg)	83.0 ± 27.1	63.9 ± 48.3	81.5 ± 81.4
Vitamin D (µg)	1.8 ± 0.6	1.5 ± 1.0	2.1 ± 1.7
Vitamin E (µg)	1.9 ± 1.1	1.5 ± 1.4	1.4 ± 0.9
Omega-3 (g)	0.37 ± 0.30	0.36 ± 0.19	0.36 ± 0.20

5.3.2. Circulating CK

CV values for all colorimetry and ELISA analysis kits are shown in Table 5.4. Resting circulating CK levels did not significantly differ between groups at baseline (PLA: 261.4 ± 241.7 U/L; EPA: 477.9 ± 687.2 U/L; Vit-D: 160.7 ± 98.5 U/L; p=0.38). There was no significant effect of immobilisation or supplement group on circulating CK levels (Figure 5.1.A and 5.2.A). CK is an enzyme expressed by various tissues and cell types and is used as a marker of skeletal muscle damage. In general populations, baseline levels of serum CK are variable with ranges from 20 to 16,000 U/L, this wide range reflects the inconsistent occurrence of genetic factors, subclinical disorders and minor injury, and physical activity status (Prelle et al., 2002). Unpublished values from our own laboratory reported resting CK values that were not significantly different between males and females (138 ± 140 U/L and 80.8 ± 56.7 U/L, respectively, p=0.097). These values were reported for a similar study population, and, therefore, supports the mixed sex population used in the current chapter. A release of cellular components is thought to occur in response to metabolic muscle disturbance. Intracellular proteolytic enzyme activity is said to occur through a cascade of events, and can increase and promote muscle protein degradation and augment cell permeability, which allows some cell contents to leak into the circulation (Huerta-Alardin et al., 2005; Khan, 2009). The current findings of no changes in circulating CK, supports the theory that declines in skeletal muscle mass that occur in human response to disuse, can largely be attributed to decreases in protein synthesis, rather than protein degradation (Glover et al., 2008). This seems to be a characteristic that separates human atrophic response to that seen in animal models such as (Loughna et al., 1987; Thomason and Booth, 1990). Hence, inferring the impact of immobilisation

between species (such as done in some of the literature; (Bar-Shai et al., 2005)) does not seem advisable (Rennie et al., 2010).

Table 5.4. Coefficient of variation values for the CK and ELISA analysis kits in the present study. * denotes greater than, and ** lower than the manufacturer's published data.

	Coefficient of variation (%)
CK	7.1 ± 6.9*
IL-6	3.7 ± 0.6*
IL-10	2.9 ± 1.6**
TNF-α	3.8 ± 0.4**
IGF-1	2.8 ± 0.6**

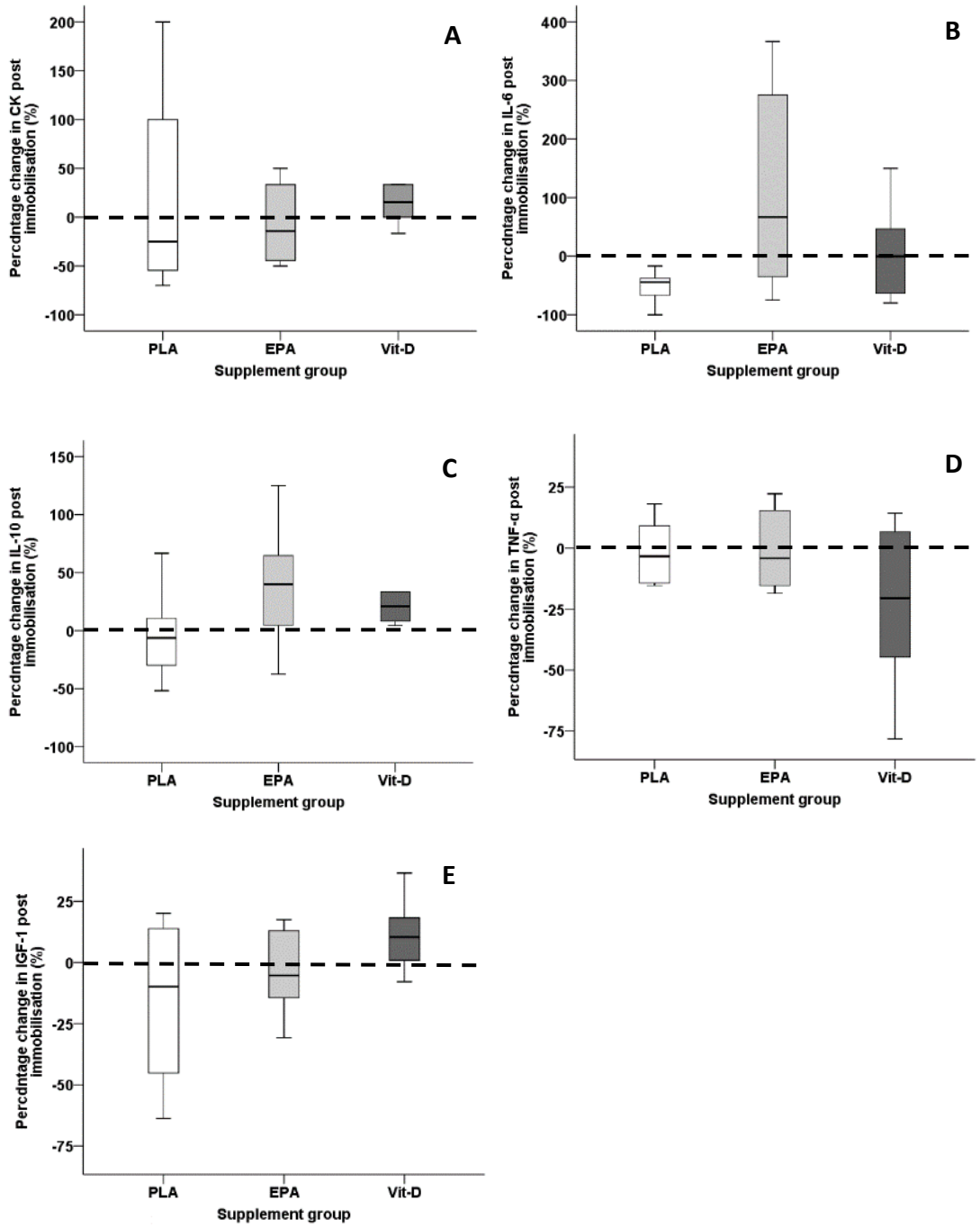

Figure 5.1. Percentage change (% ± SD) in circulating: (A) CK, (B) IL-6, (C) IL-10, (D) TNF-α and (E) IGF-1, from Pre-to-Post immobilisation for PLA, EPA and Vit-D groups. Each boxplot displays the median (middle line of the box), first quartile (bottom line of the box), third quartile (top line of the box), minimum (bottom whisker) and maximum (top whisker) values.

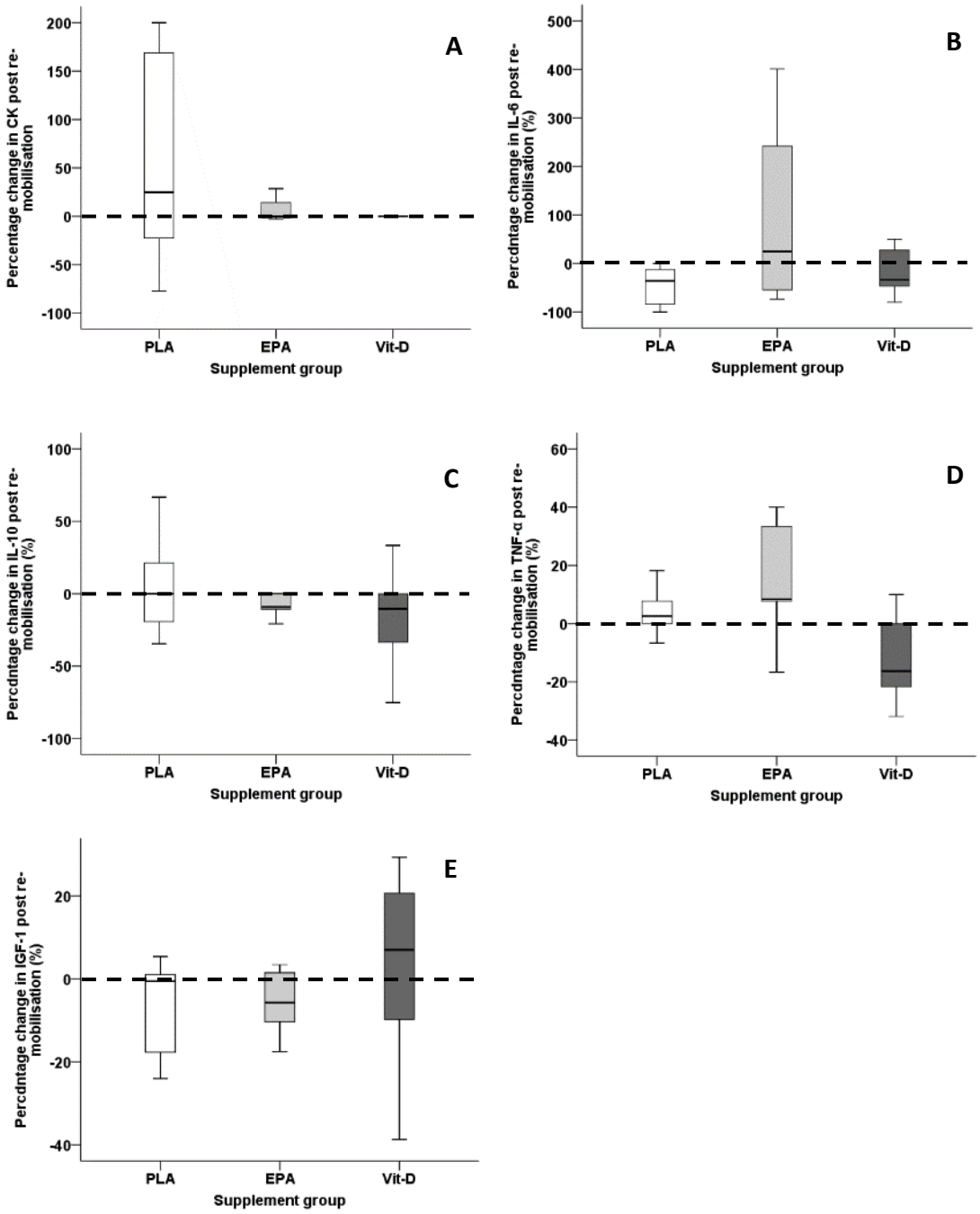

Figure 5.2. Percentage change (% ± SD) in circulating: (A) CK, (B) IL-6, (C) IL-10, (D) TNF-α and (E) IGF-1, from Pre-to-Post2 immobilisation for PLA, EPA and Vit-D groups. Each boxplot displays the median (middle line of the box), first quartile (bottom line of the box), third quartile (top line of the box), minimum (bottom whisker) and maximum (top whisker) values.

5.3.3. Circulating IL-6

Baseline circulating IL-6 levels were not significantly different between groups (PLA: 1.9 ± 1.2 pg/mL; EPA: 1.8 ± 1.7 pg/mL; Vit-D: 1.6 ± 1.3 pg/mL; p=0.93). Serum IL-6 values in the literature are similar with reports of 1.4 ± 1.0 pg/mL and 3.0 ± 0.6 pg/mL (Klausen et al., 1997; Nishimoto et al., 2008). Percentage change in circulating IL-6 levels from Pre to Post immobilisation was not significantly different between supplement groups (Figure 5.1.B and 5.2.B). IL-6 is produced and released by a wide variety of cell types, including skeletal muscle and is both a pro-inflammatory cytokine and an anti-inflammatory myokine. There are observations that IL-6 either directly or indirectly mediates catabolic effects on skeletal muscle (Goodman, 1994) and that IL-6 can directly induce skeletal muscle atrophy (Haddad et al., 2005). Drummond et al. (2013) reported increases in IL-6 mRNA in muscle samples after seven days bed-rest, whilst systemic changes were absent. The results of this and the present study suggest that relatively short-term, low total mass, disuse does not induce a change in circulating IL-6.

5.3.4. Circulating IL-10

Resting circulating IL-10 levels did not significantly differ between groups at baseline (PLA: 8.8 ± 2.2 pg/mL; EPA: 6.8 ± 2.1 pg/mL; Vit-D: 10.5 ± 6.6 pg/mL; p=0.28). In comparison to some values in the literature (1.1 ± 1.34 pg/mL) (Csuka et al., 1999), the IL-10 values reported in the present study are high. In other studies, however, median serum IL-10 values of 18.0 pg/mL (range, 0-1055 pg/mL) and 9.2 pg/mL (range, 7.4-12.0 pg/mL) have been reported in healthy control groups, which would put the current values in line with some of the literature (De Vita et al., 2000; Gupta et al., 2012). There was no significant effect of immobilisation or of supplement group on circulating IL-10 levels (Figure 5.1.C

and 5.2.C). IL-10 is a predominantly anti-inflammatory cytokine that regulates the TNF-α-converting enzyme (Schottelius et al., 1999). Deng et al. (2012) showed that IL-10 plays a central role in the regulation of normal growth and regeneration of muscle. It is well established that shifts in macrophage phenotype coincide with transitions in the stage of myogenesis in regenerating muscle (Frenette et al., 2000; McLennan, 1993; St Pierre and Tidball, 1994). That shift in macrophage phenotype is suggested to coincide with changes in expression of myogenic, regulatory transcription factors (Launay et al., 2001; McLoon et al., 1998; Yablonka-Reuveni and Rivera, 1994). Again, the present results do not demonstrate a significant change in circulating IL-10 and may suggest that IL-10 is not a key mechanism associated with non-injurious disuse-induced atrophy.

5.3.5. Circulating TNF-α

Baseline circulating TNF-α levels did not significantly differ between groups (PLA: 16.3 ± 15.2 pg/mL; EPA: 11.3 ± 7.9 pg/mL; Vit-D: 23.7 ± 21.1 pg/mL; p=0.35). The reported TNF-α levels are in line with previously reported values in the literature, with average serum TNF-α levels ranging from 10.9 to 16.8 pg/mL in mixed control populations (O'Brien et al., 2007; Pavon et al., 2006). Percentage change in circulating TNF-α levels from Pre to Post immobilisation was not significantly different between supplement groups (Figure 5.1.D and 5.2.D). TNF-α is a cytokine involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. TNF-α is one of the most prominent cytokines linked to muscle pathophysiology, being associated with muscle catabolism and loss of muscle function (Li and Reid, 2001). This study does not demonstrate significant changes in circulating TNF-α and may support previous findings of decreased protein synthesis rather than increased protein degradation in disuse-atrophy (Glover et al., 2008).

5.3.6. Circulating IGF-1

At baseline resting circulating IGF-1 levels did not significantly differ between groups (PLA: 184.7 ± 58.5 ng/mL; EPA: 252.2 ± 48.8 ng/mL; Vit-D: 221.0 ± 64.1 ng/mL; p=0.12). IGF-1 serum values ranging from 114 to 538 ng/mL are reported in the literature for a similar aged mixed sex population (Granada et al., 2000). The percentage change in IGF-1 was not significantly different between supplement groups (Figure 5.1.E and 5.2.E). IGF-1 is a protein that has anabolic effects in adults, stimulating cell growth and inhibiting cell death. It has been demonstrated that IGF-1 induces hypertrophy by stimulating the phosphatidylinositol 3-kinase (PI3k)-Akt pathway, resulting in downstream activation of proteins that are required for protein synthesis (Bodine et al., 2001; Rommel et al., 2001). The IGF-1/PI3K/Akt pathway, is therefore, identified as a crucial intracellular regulator of muscle hypertrophy (Glass, 2003b; Glass, 2003a). Several studies indicate that IGF-1 can induce hypertrophy and block atrophy (Barton et al., 2010; Barton-Davis et al., 1998; Schakman et al., 2005). The present findings do not demonstrate any changes in circulating IGF-1 levels. Observing no change in circulating levels does not rule out changes in IGF-1 in skeletal muscle itself (Sjogren et al., 1999; Yakar et al., 1999).

5.4. Conclusion

Overall, in this relatively short-term immobilisation model that utilised a small muscle mass, there appeared to be no change in circulating CK, cytokines or IGF-1 in response to immobilisation. This suggests that the atrophy and strength declines seen in such a model (see Chapters 3 and 4) are not associated with changes in the specific enzyme and hormonal factors quantified here, at least not

at the systemic level. There may be other endocrine factors, not monitored in this study that may play crucial roles in skeletal muscle atrophy.

Chapter 6: Impact of pre-immobilisation protein supplementation on the time course of immobilisation-induced atrophy and asthenia

6.1. Introduction

Previous research (summarised in Chapter 1) along with the data presented in Chapters 2, 3 and 4, show declines in skeletal muscle size, skeletal muscle strength and bone content and increases in fatty tissues content in response to disuse models. There are also several reports of the effects of disuse on muscle fatigability (Miles et al., 1994; Miles et al., 2005; Yue et al., 1997), EMG characteristics (Vaughan, 1989) and the cardiovascular system (Gayeski and Honig, 1983; Henriksson and Reitman, 1977; Shoemaker et al., 1998). The previous chapters support decreases in muscle size and strength and to some extent bone content, along with increases in fatty tissue content, with immobilisation. The data in Chapter 4 however, do not suggest significant changes in muscle fatigability, EMG characteristics or markers of cardiovascular function.

As suggested in the previous chapters, nutritional supplementation is proposed as a strong candidate for attenuating hypo-activity-induced skeletal muscle atrophy and declines in muscular strength. Looking back on all previous chapters, it is clear that the data so far highlight essential amino acid (EAA) supplementation as the most effective nutritional intervention to help prevent the deleterious changes associated with the current disuse model. Previous research (Paddon-Jones, Sheffield-Moore, Urban, et al., 2004; Stuart et al., 1990), along with Chapter 2, investigate the effects of EAA supplementation during periods of disuse. Physiological reserve refers to excess capacity in organs and the biological systems and has been defined as the buffer that allows us to cope with and recover from stressors (Kemp and Mosqueda, 2004). Whether building physiological reserve before periods of disuse (i.e. administering nutritional supplementation for a period before limb immobilisation) would have beneficial effects on the response of skeletal muscle, bone parameters and the

cardiovascular system to disuse however, is yet to be determined. The practical implications would be that for clinical orthopaedic patients, in particular those scheduled for joint replacement surgery, given that the period of hypo-activity (e.g. post-surgery) is planned, building up physiological reserve in anticipation may be an effective preventative measure against the expected atrophy/ asthenia. Indeed, previous research has shown a significant correlation between subnormal nutritional indices and the development of complications following orthopaedic surgery (Jensen et al., 1982). Similarly, in Chapter 2, there was a strong potential for liquid amino acid ingestion for reducing the impact of immobilisation.

The aim of the present study, therefore, was to determine whether pre-immobilisation supplementation with an EAA supplement before a period of limb immobilisation, would help to attenuate the declines in muscle morphology, muscle strength and bone parameters associated with upper limb immobilisation. This chapter utilises the same model of immobilisation as in Chapters 3, 4 and 5.

6.2. Methods

6.2.1. Participants

Ten healthy, recreationally active participants were recruited from the local university campus, via lecture presentations, posters and word of mouth over a one-year period. All participants gave written informed consent to participate in this study before being randomly assigned to one of two groups: placebo [PLA: n = 5] or EAA [EAA: n = 5] (anthropometric data in Table 6.1). Participants were excluded if they had any medical conditions requiring the use of medication likely to affect muscle function or musculoskeletal health. The screening process also ruled out any participants with neuro-musculoskeletal disorders that could affect muscle function. The study was in agreement with the Declaration of Helsinki and

approval was obtained from the local Ethics Committee of Manchester Metropolitan University.

Table 6.1. Baseline characteristics of all participants.

	PLA	EAA
Age (years)	23 ± 5.5	21 ± 2.9
Males	n = 2	n = 2
Females	n = 3	n = 3
Height (cm)	169.2 ± 15.8	167.6 ± 3.8
Mass (kg)	75.0 ± 20.8	68.4 ± 9.6

6.2.2. Intervention

The study used a randomised, double-blind, placebo-controlled design. At least one week prior to the first testing session all participants attended a familiarisation session. Measures of upper arm muscle and subcutaneous fat thickness, body composition (lean mass, bone content parameters and fat mass), upper and lower arm girth, isometric and isokinetic elbow torque, EMG co-contraction, muscle fatigability and arterial dimensions and blood flow were taken at every testing session. Participants attended three testing sessions in total:

1) Baseline testing session [BASE]: participants sent away with two weeks' worth of nutritional supplement
2) Second testing session [PRE]: the non-dominant arm was immobilised in a sling as per Chapters 3 and 4; in short, the sling was to be worn for a minimum of nine waking hours a day for two continuous weeks. Participants sent away with two weeks' worth of nutritional supplement

3) Third testing session [POST]: post removal of the sling and final testing session

During the BASE to PRE phase, the EAA group ingested 2 x 45 mL per day of a commercially available EAA drink (Amino Fuel Lean Muscle Liquid, Orange, Twinlab®, U.S.A.). During the BASE to PRE phase, the PLA group received a colour and taste matched placebo (Figure 6.1). Each 45 mL dose of *Amino Fuel* nutritional supplement is described by the manufacturer as containing 15 g of protein: 780 mg L-Leucine; 440 mg L-Isoleucine; 530 mg L-Valine; 760 mg L-Lysine; 560 mg L-Threonine; 200 mg L-Methionine; 320 mg L-Phenylalanine; 80 mg L-Tryptophan). Each 45 mL of the placebo mixture consisted of a homemade blend of water (35.3 mL), orange cordial (no added sugar) (7.9 mL), orange extract (1.4 mL), artificial sweeteners (0.7 Hermesetas tablets), orange and yellow food colouring (0.4 mL) and an over the counter thickening agent (2.6 g of Resource® ThickenUp™). During the PRE to POST phase (i.e. during immobilisation) both the EAA and PLA group received the colour and taste matched placebo as described above. Participants were asked to maintain their habitual diet and not to perform any unaccustomed strenuous exercise during the study duration. This was monitored as described in more detail in Chapter 3, using a 3-day food diary, a daily activity log and pedometer. These were self-reported between BASE to PRE, and between PRE to POST. The food diaries were analysed for macronutrient and micronutrient average intake using Microdiet Plus 1.2 (Microdiet, Downlee Systems Ltd, UK).

Figure 6.1.Comparison of placebo (left) and EAA (right) supplements.

6.2.3. Muscle and sub-cutaneous adipose thickness measures

Images of biceps and triceps brachii muscle and sub-cutaneous adipose tissue were obtained using B-mode ultrasonography, as previously described in Chapter 3. In short, images were obtained with the participant in an upright-seated position with their arm abducted square to the body and resting on the ultrasound machine. The midpoint (L50) and a third of the distance (L33) from the distal end of the biceps and triceps brachii were identified and marked on the skin. Images were collected in the sagittal plane at these four sites and recorded using Adobe Premiere 6.0 (Adobe Systems, USA). Muscle thickness was measured as the distance from the top of the superficial muscle aponeurosis to the bone at both sites along the biceps and triceps brachii. Sub-cutaneous adipose thickness was measured as the distance from the bottom of the epidermis to the top of the superficial muscle aponeurosis in the biceps and triceps at both sites.

6.2.4. Body composition analysis

Body composition was determined using dual-energy x-ray absorptiometry (DXA) scanner (Hologic Discovery; Vertec Scientific, Berkshire, UK), as previously

described in Chapter 3. Measures of bone mineral density (BMD), bone mineral content (BMC), lean mass, fat mass and fat percentage are reported for the immobilised arm only.

6.2.5. Arm girths

As previously described in Chapter 3, a measuring tape was used to measure upper arm girth at the mid-acromial-radial and lower arm girth at a fixed point a third of the way (proximal to the olecranon) along the length of the radiale-stylion. Arm girth measurements were taken with participants in a relaxed standing position with arms hanging by the sides and palms facing the hips (i.e. anatomical zero).

6.2.6. Isometric dynamometry

In depth dynamometry details are reported in Chapter 4. Briefly, isometric elbow torque was assessed using a Cybex dynamometer (Cybex, New York, USA). Two repetitions of isometric contractions were performed at six different elbow joint angles (60°, 70°, 80°, 90°, 100° and 110°), 60 seconds apart. Participants were instructed to rapidly exert maximal torque against the dynamometer lever arm over a 3-4 second period. First in flexion and, five seconds after return to baseline, in extension. The highest torque (peak torque averaged over a 500 ms period either side of instantaneous peak) from the repeated efforts, was recorded as the participant's maximal voluntary contraction (MVC) for each angle.

6.2.7. Isokinetic dynamometry

Three continuous elbow extension and flexion isokinetic contractions were completed at six different randomised order speeds (30, 60, 90, 120, 180 and 240°/sec), separated by 90 seconds. The highest of the three consecutive efforts

at each speed was recorded as peak isokinetic torque (25 ms either side of the instantaneous peak) for elbow extension and flexion.

6.2.8. Electromyographic measurements

During the isometric and isokinetic contractions, muscle recruitment level and patterns were assessed using electromyography (EMG) as described in Chapter 4. Percentage biceps co-contraction was calculated (biceps EMG during extension / biceps EMG during flexion) for both isometric and isokinetic contractions. Percentage triceps co-contraction was also calculated (triceps EMG during flexion / triceps EMG during extension) again for both isometric and isokinetic contractions.

6.2.9. Fatiguing contractions

Maximal isometric contractions were performed for 30 seconds with the dynamometer lever arm locked at a 90° angle (where 180° is full elbow extension) in the direction of elbow flexion, and after 90 seconds recovery, in the direction of elbow extension. The mean, slope and standard deviation of the torque trace were recorded through the 30-second contractions. Fast Fourier transform (FFT) was computed for the agonist muscle during the first five and last five seconds of each fatiguing contraction. Median frequency values were determined for these time points and a change in median frequency (last 5 minutes – first 5 minutes / first 5 minutes) was then computed as a measure of the fatigability of the muscle.

6.2.10. Arterial resting blood flow

Measurements of resting brachial artery diameter, heart rate (HR), resistance index (RI) and flow by diameter (FbD) were obtained using an echo Doppler ultrasound machine (AU5, Esaote, Genoa, Italy). FbD was a measure of velocity

(m/s). The values were obtained in the sagittal plane in line with the marker of the midpoint of the biceps brachii (as previously marked earlier for obtaining muscle ultrasound images). For more details, please refer to Chapter 4.

6.2.11. Statistics

Data were analysed using IBM SPSS v21 (IBM Inc, USA). The Shapiro-Wilk test revealed some of the data to be non-parametric (muscle and sub-cutaneous adipose thickness, body composition, isometric and isokinetic co-contraction, FFT and arterial resting blood flow). Percentage change relative to baseline values (BASE-to-PRE: (PRE-BASE)/BASE; and BASE-to-POST: (POST-BASE)/BASE) was calculated for all variables. Parametric data were analysed using a mixed design 2x2 ANOVA (where the within factor is time phase: BASE-to-PRE and BASE-to-POST; and the between factor is supplement group: PLA vs. EAA), using the percentage change data. Non-parametric data were analysed in two parts: a) within group effects was analysed using Friedman tests on raw data (BASE, PRE and POST) and the Wilcoxon signed rank test to compare the percentage change values within groups; b) between group effect was analysed using Mann-Whitney U tests to compare percentage change values between groups. All data are presented as mean ± standard deviation (SD). Statistical significance was set with alpha at < 0.05.

6.3. Results

6.3.1. Daily physical activity and nutritional intake

There was no significant change in self-reported nutritional intake from the period before immobilisation compared to during immobilisation in either supplement group. Nutritional intake was not significantly different between groups at either phase (Table 6.2). EAA supplementation during the pre-immobilisation phase

resulted in a significantly greater protein intake before immobilisation in the EAA group than during immobilisation ($p=0.027$) and a significantly greater protein intake compared to the PLA group ($p<0.048$). There was no significant difference in habitual physical activity before, compared to during immobilisation or between supplement groups (Table 6.2).

6.3.2. Muscle and sub-cutaneous adipose thickness measures

Muscle and sub-cutaneous adipose thickness were not significantly different between groups at baseline (muscle thickness: bicep L50 $p=0.144$; bicep L33 $p=0.600$; triceps L50 $p=0.595$; triceps L33 $p=0.629$ and sub-cutaneous adipose thickness: bicep L50 $p=0.718$; bicep L33 $p=0.508$; triceps L50 $p=0.815$; triceps L33 $p=0.962$) (Table 6.3). There was a significant decrease in muscle thickness (bicep L50 $p=0.010$; bicep L33 $p=0.004$; triceps L50 $p=0.019$; triceps L33 $p=0.012$) and a significant increase in sub-cutaneous adipose thickness (bicep L50 $p=0.037$; bicep L33 $p=0.020$; triceps L50 $p=0.044$; triceps L33 $p=0.033$) at all sites on the biceps and triceps brachii POST immobilisation. There was no significant change in any muscle or sub-cutaneous thickness measures after supplementation from BASE to PRE immobilisation (muscle thickness: bicep L50 $p=0.905$; bicep L33 $p=0.636$; tricep L50 $p=0.100$; triceps L33 $p=0.100$ and sub-cutaneous adipose thickness: bicep L50 $p=1.000$; bicep L33 $p=0.72$; triceps L50 $p=0.464$; triceps L33 $p=0.423$). The relative change values in muscle thickness and sub-cutaneous adipose thickness were not significantly different between the PLA and EAA groups (muscle thickness: bicep L50 $p=0.548$; bicep L33 $p=0.421$; triceps L50 $p=0.548$; triceps L33 $p=0.421$ and sub-cutaneous adipose thickness: bicep L50 $p=0.690$; bicep L33 $p=0.841$; triceps L50 $p=0.841$; triceps L33 $p=0.690$) (Figure 6.2).

Table 6.2. Nutritional intake and daily physical activity before immobilisation and during immobilisation. * significant difference between before and after immobilisation. † significant difference between supplement groups.

	Before immobilisation		During immobilisation	
	PLA	EAA	PLA	EAA
Calorie intake (kcal/day)	1654± 428	1887 ± 292	1920 ± 409	1524 ± 301
Protein intake ($g \cdot kg^{-1} \cdot bw/day$)	1.0 ± 0.2	0.9± 0.3	1.3 ± 0.3	1.0 ± 0.4
Protein intake + supplement ($g \cdot kg^{-1} \cdot bw/day$)	1.0 ± 0.2†	1.4 ± 0.3*†	1.3 ± 0.3	1.0 ± 0.4*
Carbohydrate intake ($g \cdot kg^{-1} \cdot bw/day$)	2.4 ± 0.8	3.3 ± 0.8	2.8 ± 1.1	2.6 ± 0.8
Fat ($g \cdot kg^{-1} \cdot bw/day$)	1.0 ± 0.3	1.3 ± 0.5	1.2 ± 0.4	1.0 ± 0.6
Habitual physical activity (steps/day)	7032 ± 1264	7612 ± 4262	6946 ± 2355	7415 ± 3274

Table 6.3. Baseline biceps and triceps muscle and sub-cutaneous thickness measures.

	PLA	EAA
Biceps muscle thickness (L50) (mm)	34.5 ± 5.3	30.0 ± 3.4
Biceps muscle thickness (L33) (mm)	31.3 ± 6.1	29.6 ± 3.9
Triceps muscle thickness (L50) (mm)	44.4 ± 4.4	43.2 ± 2.3
Triceps muscle thickness (L33) (mm)	45.2 ± 4.7	44.0 ± 2.5
Biceps sub-cutaneous adipose thickness (L50) (mm)	5.5 ± 4.0	4.7 ± 3.2
Biceps sub-cutaneous adipose thickness (L33) (mm)	4.8 ± 1.9	3.9 ± 2.0
Triceps sub-cutaneous adipose thickness (L50) (mm)	9.5 ± 9.4	8.4 ± 2.8
Triceps sub-cutaneous adipose thickness (L33) (mm)	7.6 ± 6.3	7.4 ± 3.4

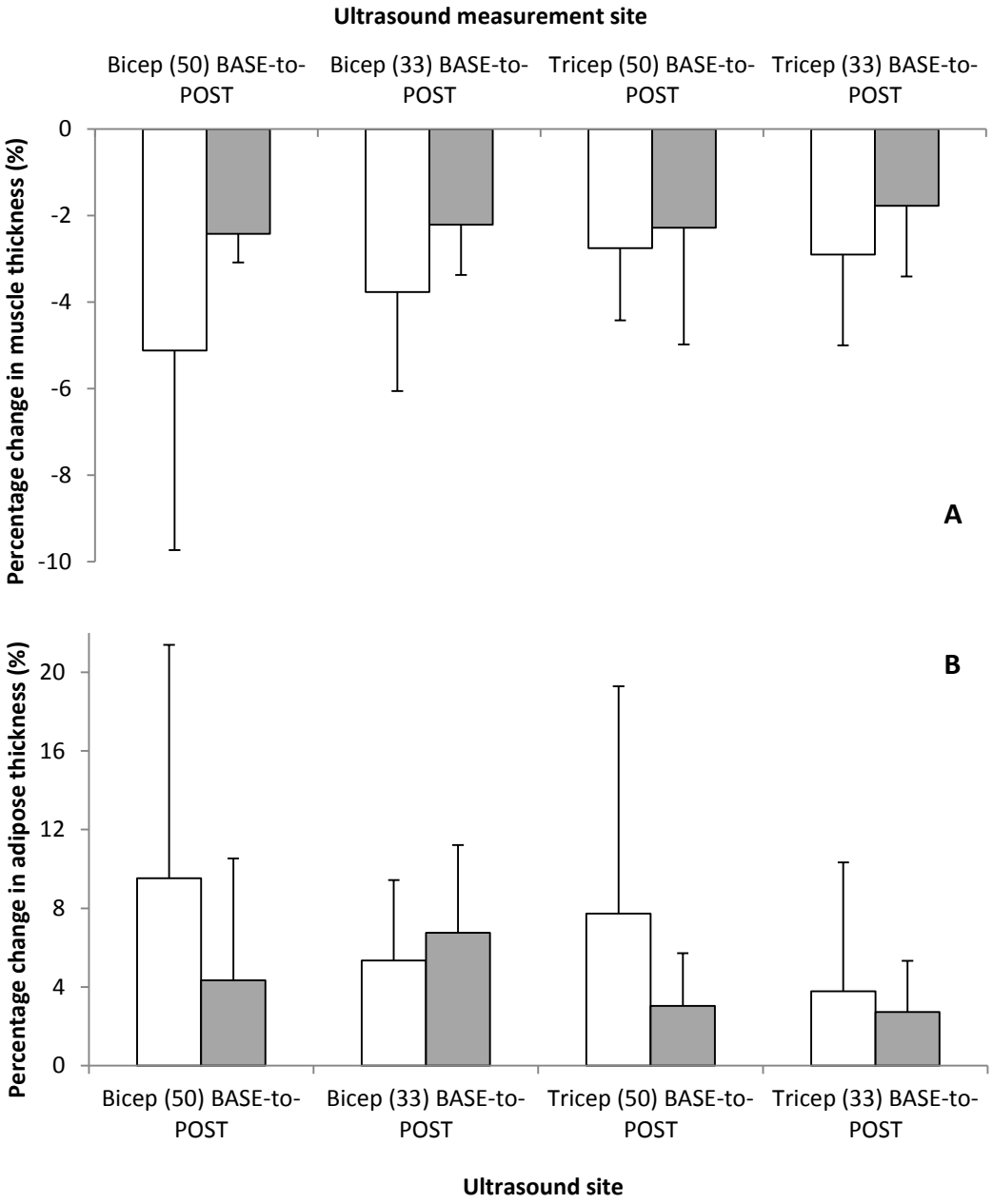

Figure 6.2. Percentage change (% ± SD) in, (A) muscle thickness and (B) sub-cutaneous adipose thickness, from BASE-to-POST immobilisation for PLA (white bars) and EAA (grey bars) at the midpoint (L50) and a third of the distance (L33) along the length of the biceps and triceps brachii.

6.3.3. Arm girths

Baseline upper arm girth (PLA: 29.6 ± 4.7 mm; EAA: 27.4 ± 1.9 mm) and lower arm girth (PLA: 25.6 ± 2.9 mm; EAA: 25.3 ± 1.4 mm) were not significantly different between the two groups (p=0.357 and p=0.801, respectively). There was a significant decrease in upper and lower arm girth POST immobilisation (p=0.001 and p=0.003, respectively), with no significant difference between groups in the relative change values (p= 0.894 and p=0.523, respectively) (Figure 6.3).

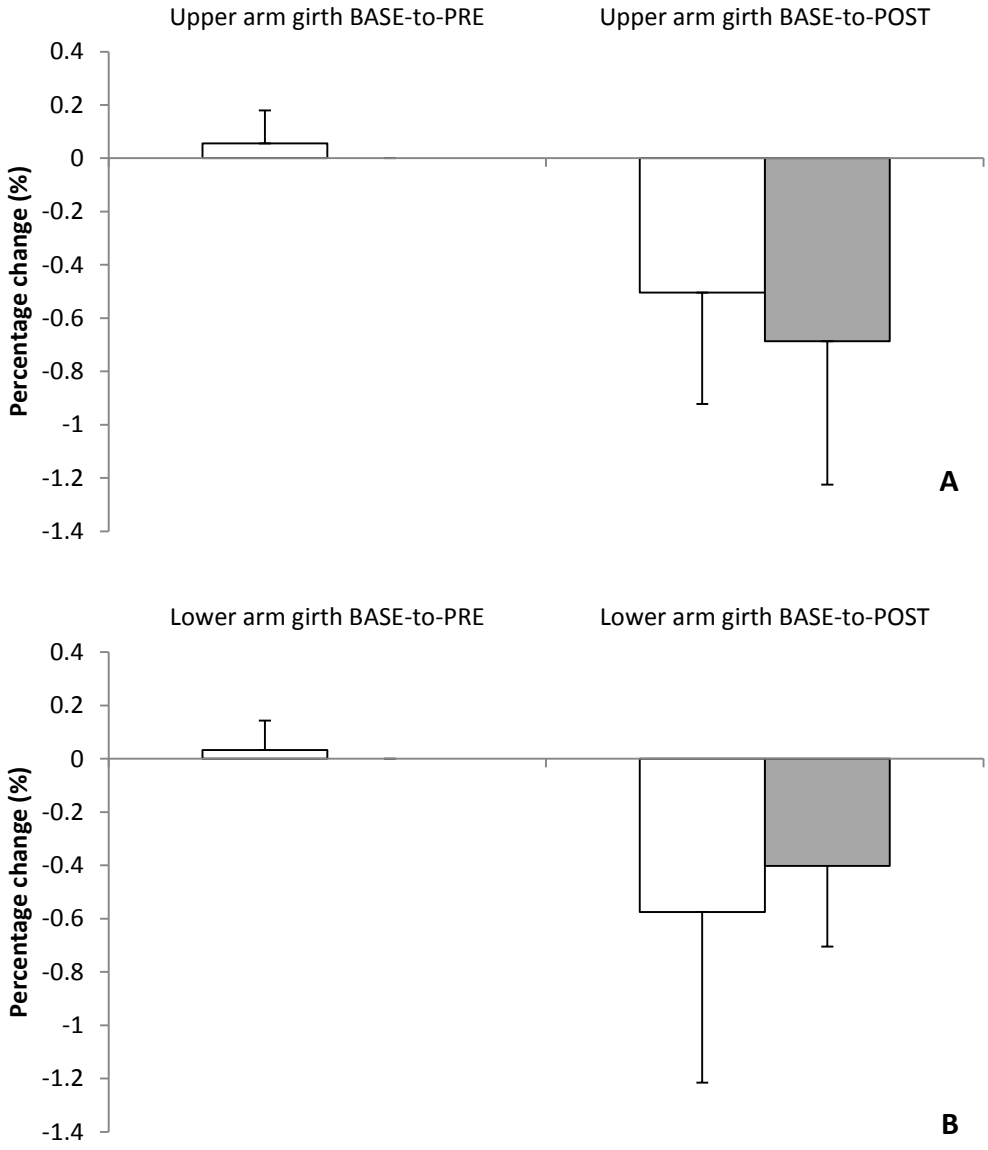

Figure 6.3. Percentage change (% ± SD) in upper (A) and lower (B) arm girth from BASE-to-PRE and BASE-to-POST for PLA (white bars) and EAA (grey bars).

6.3.4. Body composition

Baseline lean mass (PLA: 2364.7 ± 982.1 g; EAA: 2271.7 ± 511.0 g), BMD (PLA: 0.8 ± 0.1 g/cm^2; EAA: 0.8 ± 0.1 g/cm^2), BMC (PLA: 167.1 ± 51.8 g; EAA: 169.7 ± 38.8 g), fat mass (PLA: 1288.6 ± 583.9 g; EAA: 1180.0 ± 496.4 g) and fat percentage (PLA: 34.0 ± 12.7 %; EAA: 31.5 ± 10.3 %) were not significantly different between supplement groups (p=0.934; p=0.771; p=0.850, p=0.521, p=0.485, respectively). Lean mass decreased significantly POST immobilisation (p=0.34), with no significant difference between groups in relative change values (p=0.693) (Figure 6.4). There was no significant change in BMD, BMC, fat mass nor fat percentage throughout supplementation or immobilisation (p=0.275; p=0.566, p=0.125, p=0.737 respectively). Similarly, there was no significant effect of supplementation group on relative change values (p=0.596; p=0.654, p=0.781, p=0.332, for BMD, BMC, fat mass and fat percentage, respectively) (Table 6.4).

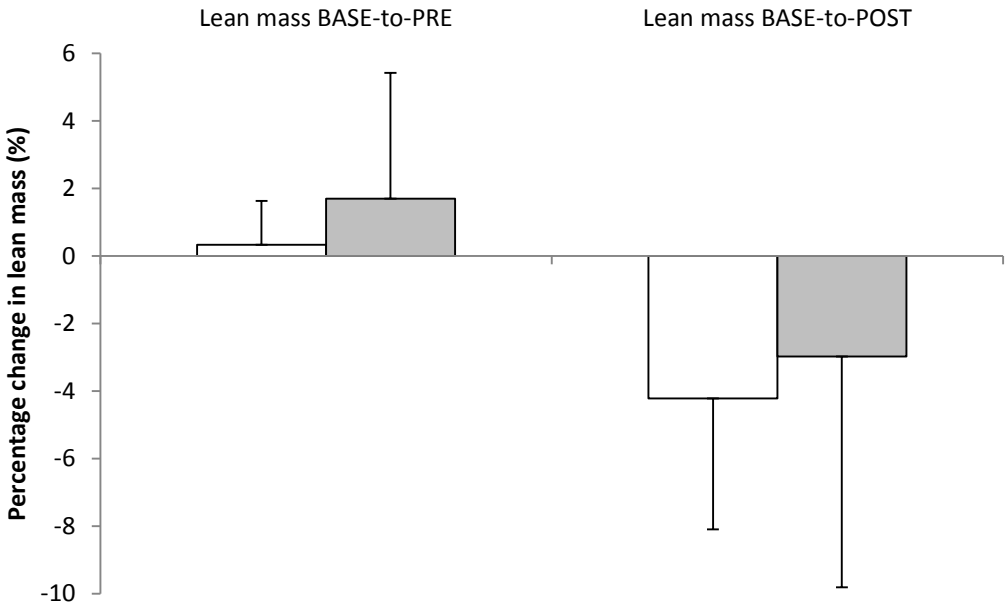

Figure 6.4. Percentage change (% ± SD) in lean mass from BASE-to-PRE and BASE-to-POST for PLA (white bars) and EAA (grey bars).

Table 6.4. Percent changes (% ± SD) in immobilised limb composition in response to immobilisation and supplementation.

	PLA		EAA	
	BASE-to-PRE	BASE-to-POST	BASE-to-PRE	BASE-to-POST
BMD	0.3 ± 2.6	-0.1 ± 0.4	2.4 ± 2.8	3.1 ± 2.1
BMC	0.2 ± 0.2	-0.3 ± 1.5	0.6 ± 1.4	-0.4 ± 1.0
Fat mass	1.0 ± 0.6	3.2 ± 3.5	0.5 ± 1.0	1.9 ± 3.2
Fat %	0.6 ± 2.8	1.7 ± 3.8	1.2 ± 9.0	-3.0 ± 7.7

6.3.5. Isometric dynamometry

Isometric MVC elbow flexion and extension torque were not significantly different between groups at baseline (flexion: 60° p=0.909; 70° p=0.776; 80° p=0.375; 90° p=0.882; 100° p=0.723; 110° p=0.897 and extension: 60° p=0.386; 70° p=0.410; 80° p=0.300; 90° p=0.360; 100° p=0.584; 110° p=0.235). Isometric MVC torque decreased from BASE to POST immobilisation, at every angle for elbow flexion (60° p=0.0002; 70° p=0.015; 80° p=0.003; 90° p=0.001; 100° p=0.035) and elbow extension (60° p=0.039; 80° p=0.026; 90° p=0.043), except for flexion at 110° (p=0.065) and extension at 70° (p=0.204), 100° (p=0.109) and 110° (p=0.173) (Figure 6.5). Average isometric torque decrease from BASE to POST immobilisation across angles for flexion were -14.0 ± 2.7 % and -12.5 ± 2.4 %, and for extension were -11.3 ± 3.0 % and -10.7 ± 4.2 %, for PLA and EAA, respectively. There was no significant effect of supplement group on relative percentage change values for any of the isometric elbow torque measurements (flexion: 60° p=0.162; 70° p=0.965; 80° p=0.898; 90° p=0.819; 100° p=0.998; 110°

p=0.974 and extension: 60° p=0.946; 70° p=0.994; 80° p=0.770; 90° p=0.751; 100° p=0.768; 110° p=0.859).

6.3.6. Isokinetic dynamometry

Isokinetic MVC elbow flexion and extension torque were not significantly different between groups at baseline (flexion: 30°/sec p=0.971; 60°/sec p=0.764; 90°/sec p=0.881; 120°/sec p=0.826; 180°/sec p=0.427; 240°/sec p=0.742 and extension: 30°/sec p=0.974; 60°/sec p=0.766; 90°/sec p=0.744; 120°/sec p=0.377; 180°/sec p=0.705; 240°/sec p=0.735). Isokinetic torque significantly decreased POST immobilisation at all speeds for both flexion (30°/sec p=0.006; 60°/sec p=0.016; 90°/sec p=0.011; 120°/sec p=0.024; 180°/sec p=0.008; 240°/sec p=0.017) and extension (30°/sec p=0.009; 60°/sec p=0.006; 90°/sec p=0.042; 120°/sec p=0.004; 180°/sec p=0.044; 240°/sec p=0.022) (Figure 6.6). Average isokinetic torque decrease from BASE to POST immobilisation across speeds for flexion were -9.3 ± 3.5 % and -10.5 ± 3.1 %, and for extension were -12.8 ± 3.9 % and -7.6 ± 2.4 %, for PLA and EAA, respectively. There was no significant effect of supplement group on relative percentage change values for any of the isokinetic elbow torque measurements (flexion: 30°/sec p=0.209; 60°/sec p=0.919; 90°/sec p=0.806; 120°/sec p=0.603; 180°/sec p=0.990; 240°/sec p=0.889 and extension: 30°/sec p=0.976; 60°/sec p=0.116; 90°/sec p=0.693; 120°/sec p=0.177; 180°/sec p=0.114; 240°/sec p=0.129).

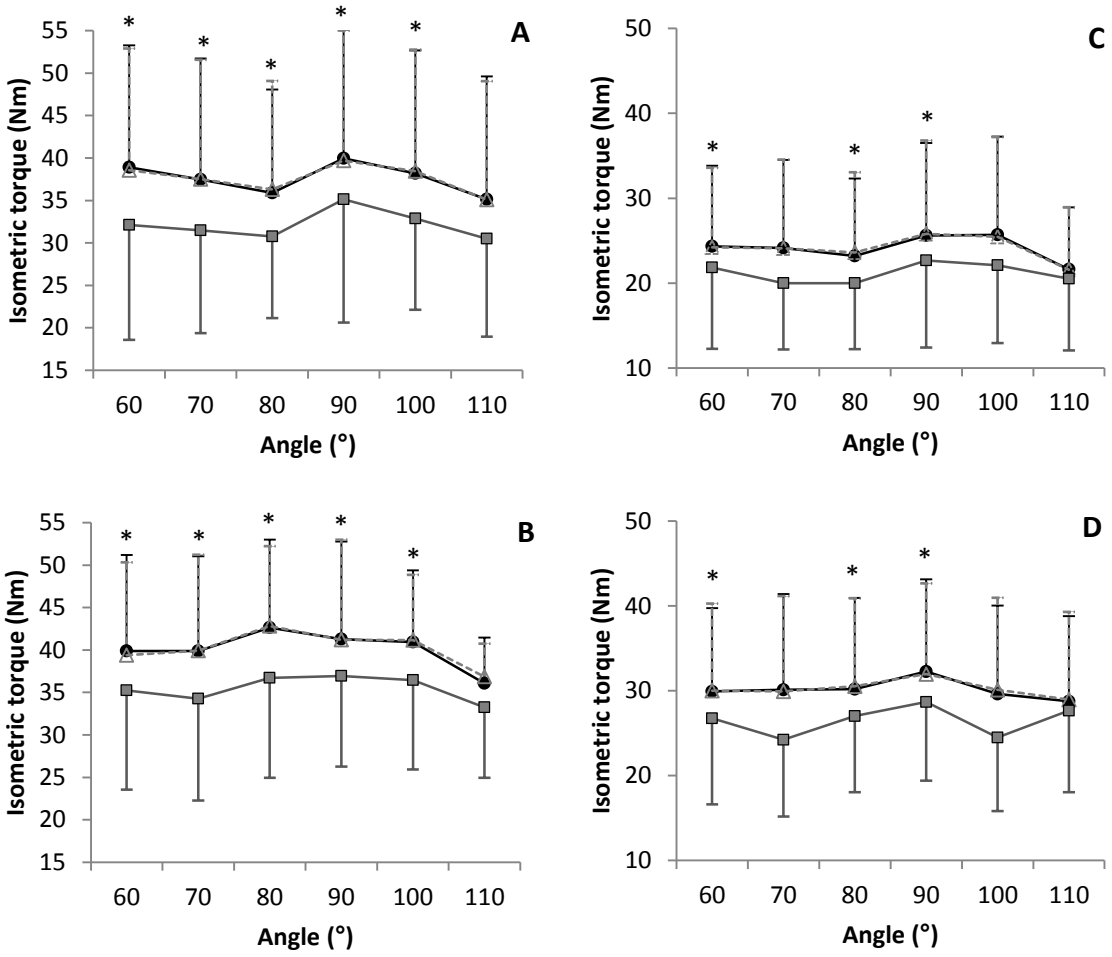

Figure 6.5. BASE (circles), PRE (triangles) and POST (squares) values for isometric torque (Nm ± SD) for elbow flexion (A = PLA; B = EAA) and elbow extension (C = PLA; D = EAA) at the six different joint angles (60-110°). * significant decrease from BASE to POST immobilisation.

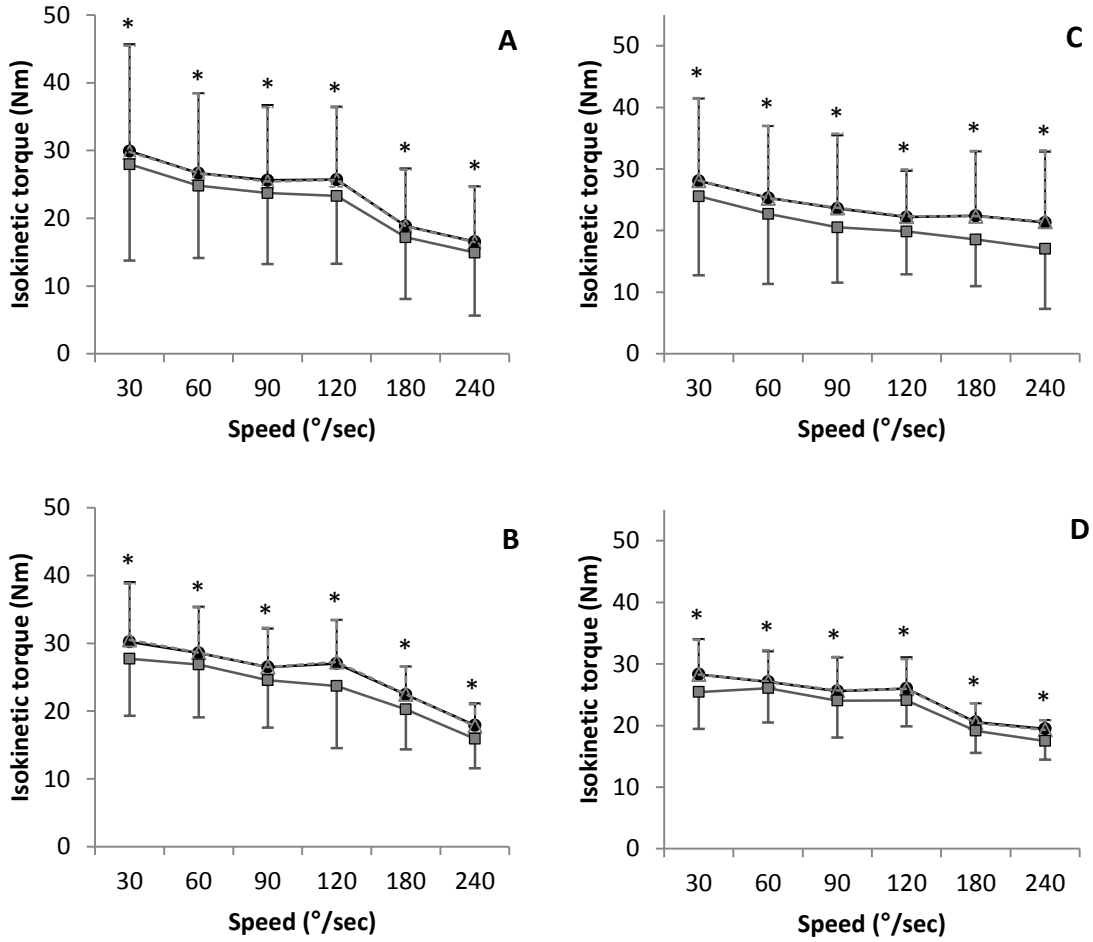

Figure 6.6. BASE (circles), PRE (triangles) and Post2 (squares) values for isokinetic torque (Nm ± SD) for elbow flexion (A = PLA; B = EAA) and elbow extension (C = PLA; D = EAA) at the six different speeds (30-240°/sec). * significant decrease from BASE to POST immobilisation.

6.3.7. Electromyographic measurements

Analysis of biceps and triceps co-contraction RMS EMG values during the isometric contractions showed no significant changes from BASE to PRE and BASE to POST immobilisation and no effect of supplement group on the relative percentage changes ($p>0.05$). Average percentage change in co-contraction RMS EMG values during isometric contractions decrease from BASE to POST immobilisation across angles for biceps were -0.22 ± 0.39 % and 0.05 ± 0.08 %, and for triceps were -0.09 ± 0.08 % and -0.04 ± 0.06 %, for PLA and EAA, respectively. Similarly, analysis of the co-contraction RMS EMG values during the isokinetic contractions displayed no significant effect of immobilisation nor of supplement group on the EMG response to the immobilisation ($p>0.05$). Average co-contraction RMS EMG values during isokinetic contractions decrease from BASE to POST immobilisation across speeds for biceps were 0.30 ± 0.42 % and -0.16 ± 0.11 %, and for triceps were -0.07 ± 0.08 % and -0.02 ± 0.08 %, for PLA and EAA, respectively.

6.3.8. Fatiguing contractions

The mean, slope and standard deviation of the torque traces showed no significant changes with immobilisation phase for either flexion or extension fatiguing contractions ($p>0.05$) (Table 6.5). There was no effect of supplement group on percentage changes in the mean, slope or standard deviation of the torque trace. Analysis of the FFT of EMG traces of the biceps and triceps brachii revealed no significant effect of immobilisation or supplementation group on rate coding change from the beginning (first 5 sec of trace) to the end (last 5 sec of trace) of a fatiguing maximal isometric contraction ($p>0.05$).

Table 6.5. Fatigue characteristics in response to immobilisation and supplementation.

		PLA	EAA
Flexion mean torque (Nm)	BASE	27.1 ± 10.7	27.6 ± 6.4
	PRE	27.7 ± 9.8	28.2 ± 3.5
	POST	25.0 ± 9.2	25.7 ± 8.0
Flexion standard deviation	BASE	2.1 ± 0.6	2.9 ± 1.5
	PRE	2.6 ± 0.6	3.1 ± 1.9
	POST	2.1 ± 0.5	2.3 ± 1.1
Flexion slope	BASE	-0.23 ± 0.11	-0.23 ± 0.25
	PRE	-0.19 ± 0.11	-0.23 ± 0.22
	POST	-0.20 ± 0.10	-0.20 ± 0.16
Flexion agonist FFT 1st five seconds (Hz)	BASE	106.1 ± 8.2	106.8 ± 13.1
	PRE	107.5 ± 7.4	107.0 ± 13.1
	POST	110.2 ± 4.9	101.5 ± 11.8
Flexion agonist FFT last five seconds (Hz)	BASE	105.5 ± 5.7	106.1 ± 11.4
	PRE	107.0 ± 4.6	106.0 ± 11.4
	POST	111.4 ± 7.7	102.2 ± 8.6
Extension mean torque (Nm)	BASE	19.2 ± 8.6	25.2 ± 9.1
	PRE	19.9 ± 8.7	26.0 ± 10.9
	POST	19.1 ± 8.0	20.8 ± 7.0
Extension standard deviation	BASE	1.4 ± 0.7	2.8 ± 1.3
	PRE	1.2 ± 0.7	2.0 ± 1.3
	POST	1.5 ± 0.9	1.9 ± 0.8
Extension slope	BASE	-0.09 ± 0.10	-0.10 ± 0.09
	PRE	-0.11 ± 0.10	-0.06 ± 0.07
	POST	-0.07 ± 0.06	-0.14 ± 0.09
Extension agonist FFT 1st five seconds (Hz)	BASE	121.0 ± 14.7	123.4 ± 9.7
	PRE	118.7 ± 16.4	126.1 ± 12.1
	POST	115.2 ± 20.7	121.9 ± 7.9
Extension agonist FFT last five seconds (Hz)	BASE	119.1 ± 16.6	123.4 ± 9.5
	PRE	118.6 ± 15.6	123.6 ± 10.7
	POST	117.6 ± 16.7	118.8 ± 6.9

6.3.9. Arterial resting blood flow

Baseline brachial artery diameter (PLA: 2.9 ± 0.5 mm; EAA: 2.6 ± 0.1 mm), HR (PLA: 72.5 ± 17.3 bpm; EAA: 70.0 ± 9.4 bpm), RI (PLA: 0.8 ± 0.1; EAA: 0.6 ± 0.3) and FbD (PLA: 0.09 ± 0.01 m/sec; EAA: 0.07 ± 0.01 m/sec), were not significantly different between supplement groups (Table 6.6.b). There was no significant change in brachial artery diameter, HR, RI and FbD from BASE to PRE nor BASE to POST immobilisation ($p>0.05$). Similarly, there was no significant effect of supplement group on the relative percent changes in these parameters ($p>0.05$) (Table 6.6.a).

Table 6.6.a. Percent change (% ± SD) in blood kinetic parameters in response to immobilisation and supplementation.

	PLA		EAA	
	BASE-to-PRE	BASE-to-POST	BASE-to-PRE	BASE-to-POST
Brachial artery diameter	0.2 ± 0.3	-0.6 ± 1.3	2.2 ± 3.7	0.2 ± 0.4
HR	-1.4 ± 1.5	2.8 ± 2.7	0.9 ± 3.2	4.6 ± 5.4
RI	-0.7 ± 2.2	6.2 ± 10.8	7.3 ± 11.4	19.4 ± 16.9
FbD	3.6 ± 17.4	-6.3 ± 11.5	-1.8 ± 6.2	-7.5 ± 25.0

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Table 6.6.b. Blood kinetic parameters (mean ± SD) at BASE, PRE and POST immobilisation in the PLA and EAA groups.

	PLA			EAA		
	BASE	PRE	POST	BASE	PRE	POST
Brachial artery diameter (mm)	2.9 ± 0.5	2.9 ± 0.5	2.9 ± 0.5	2.6 ± 0.1	2.8 ± 0.3	2.6 ± 0.2
HR (bpm)	72.5 ± 17.3	71.4 ± 17.0	74.5 ± 17.9	70.0 ± 9.4	71.0 ± 13.2	73.1 ± 10.0
RI	0.8 ± 0,1	0.8 ± 0.1	0.8 ±0.1	0.6 ± 0.3	0.6 ± 0.3	0.7 ± 0.3
FbD (m/s)	0.09 ± 0.01	0.09 ± 0.02	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01

6.4. Discussion

The purpose of this study was to determine whether pre-immobilisation supplementation with an EAA supplement before a period of limb immobilisation, would help to attenuate the declines in muscle morphology, muscle strength and bone parameters associated with upper limb immobilisation. As in the previous chapters, there was a significant change in muscle thickness, sub-cutaneous adipose thickness, arm girth, lean mass and isometric and isokinetic elbow flexion and extension torque following immobilisation. Also in line with the previous chapters, this immobilisation model had no impact on the assessed co-contraction and muscle fatigability, or on the assessed blood flow parameters. Despite significant immobilisation-induced changes in muscle size and strength and in adiposity, there was no significant impact of pre-immobilisation EAA supplementation.

Pre-immobilisation supplementation with the current dosage of EAA was not successful at attenuating the disuse-induced changes in muscle size and strength. Despite the EAA supplement causing a significant increase in total protein intake in the EAA group (compared to that seen in the PLA group). It may be that the protein intake of all participants was high enough without any supplementation (Trumbo et al., 2002). EAAs are those that cannot be synthesised by the body itself. The body maintains a small reserve of amino acids for use as required; this is called the amino acid pool. Those lacking in dietary amino acid intake may not have a sufficient amino acid pool, and therefore, intervention is needed. The young and healthy participants used in the current chapter however, are less likely to have an insufficient amino acid pool.

The dietary analysis of the participants in the current study would suggest that they had adequate nutritional intake and that malnutrition is unlikely

(Karsegard et al., 2004). It is possible that in populations where malnutrition is present, that pre-loading, and therefore, increasing physiological reserve, would be of benefit. Previous research has shown that pre-operative nutritional status can affect factors such as healing, rate of infection and length of hospital stay (Greene et al., 1991; Jevsevar and Karlin, 1993; Dempsey et al., 1988). Therefore, populations with malnutrition, who are known to be undergoing periods of hypo-activity/disuse may benefit from nutritional supplementation before this event. This is of particular relevance for older populations in which nutritional intake is known to be less adequate. Future research is warranted in to the effects of pre-immobilisation supplementation in at risk groups.

6.5. Conclusion

In summary, upper limb immobilisation resulted in a decrease in biceps and triceps brachii muscle thickness, arm girth, lean mass, and elbow isometric and isokinetic torque. The immobilisation model also resulted in a significant increase in biceps and triceps brachii sub-cutaneous adipose thickness, with no observed effect on co-contraction, muscle fatigability or resting blood flow. No significant effect of pre-immobilisation EAA supplementation on any of these parameters were observed.

Chapter 7: Summary, conclusions and recommendations

7.1. Summary

The aim of this thesis was to examine the impact of nutritional supplementation through essential amino acids (EAA), omega-3 (ω-3) or vitamin D on the deleterious physiologic impact of limb immobilisation.

The review of the current literature (Chapter 1) highlighted to the reader the profound changes in skeletal muscle morphology and strength with hypo-activity models. Losses of muscle mass and strength vary between different hypo-activity models, with immobilisation causing the most profound decreases. An immobilisation model was therefore, selected for use throughout the current thesis. The current literature subsequently showed that immobilisation of the upper limb was relatively less investigated compared to the lower limb, and therefore, provided an opportunity to increase research in this area. The literature review also highlighted the potential for nutritional supplementation as a viable intervention to combat muscle atrophy with hypo-activity, when exercise is not a practical prescription. The literature review discussed several potential nutritional supplements that could be used to combat muscle atrophy. It is evident that extensive research is needed to determine the most affective supplement and this lead to the choice of three supplements (EAA, ω-3 and vitamin D) to be investigated for their potential as an intervention to prevent the associated changes with limb immobilisation.

The starting point (Chapter 2) of the data section of this thesis was to determine the role that EAA supplementation played in attenuating atrophy induced through a model that would emulate relatively short-term decreased local mobility/activity in humans. Studies using EAA as a nutritional intervention in immobilisation are scarce in the literature. It was hypothesised that EAA supplementation during combined shoulder and arm immobilisation would

attenuate the deleterious changes associated with disuse models. This hypothesis was partly supported, in that arm girth decreased more so with the placebo supplement than the EAA supplement and with the suggestion of a greater decrease in elbow flexion and extension torque, as well as significantly further increased sub-cutaneous fatty tissue with the placebo supplement. The decreases in muscle thickness (only significant in the triceps brachii) do not explain the differential arm girth responses between EAA and PLA groups. The multi-ingredient nature of the supplement makes it difficult to identify which specific amino acid or combination thereof, may have been responsible for the observed changes. Based on the literature, the likelihood is that leucine was the main effector (which has been reported in a recent review by Breen and Phillips (2012)). Elbow flexion Torque normalised by muscle thickness in the biceps decreased less in the EAA compared with the CON group (-6 % vs. -20 %, $p<0.05$). Similarly, normalised elbow extension torque in the triceps also changed differently in the EAA compared with the CON group (+15 % vs. -23 %, $p<0.05$). Chapter 2 revealed that EAA supplementation impacts positively on the immobilisation-induced changes in the structural and functional characteristic of the remaining muscle.

The immobilisation model used in Chapter 2 could have relevance to both sporting (e.g. off-season detraining modulation) as well as clinical (e.g. injury/illness induced short-term immobilisation/bed-rest) populations. The current results warrant future research in arm immobilisation of similar duration. In this earlier chapter, although changes in muscle thickness were observed in response to sling immobilisation, the data analysis was limited to a single site ultrasound scan, half way along the muscle length. This partial analysis may have inflated the overall impact of the immobilisation as it is known that muscle immobilisation alters the functional length of the muscle (Tabary et al., 1972). It is therefore, proposed

that multiple sites be monitored along the length of the muscle, so that the overall impact of EAA supplementation (e.g. on elbow flexors/ extensors) may be further clarified.

Following on from the effect of EAA supplementation (Chapter 2), the purpose of the following three chapters (Chapter 3, 4 and 5) was to determine the role that two potential protein-sparing modulators (ω-3 or vitamin D supplementation) may play in attenuating the deleterious physiological changes induced through 2 weeks of 9-waking-hours-per-day combined arm and shoulder immobilisation. Chapter 3 focussed on the modulation of appendicular mass content, composition and structure. It was hypothesised that muscle thickness, lean mass and arm girth would decrease with limb immobilisation. In accordance with this hypothesis and with previous research (Miles et al., 1994; Veldhuizen et al., 1993; Yue et al., 1997) muscle thickness (-5.4 ± 4.3 %), upper and lower arm girth (-1.3 ± 0.4 % and -0.8 ± 0.8 %, respectively) and lean mass (-3.6 ± 3.7 %) decreased significantly with limb immobilisation in the placebo group ($p<0.05$). Changes in bone mineral content (BMC) and bone mineral density (BMD) are often found in longer and more severe periods of disuse (Marchetti et al., 1996; Rittweger et al., 2005; Rittweger et al., 2009). Somewhat surprisingly, therefore, we observed a significant decrease in BMC (-2.3 ± 1.5 %) with this limited degree/duration of limb immobilisation ($p<0.05$). The BMD and BMC values reported in Chapter 3 may have been influenced by the observed changes in muscle and sub-cutaneous adipose thickness. Previous research indicates that BMD is affected by changes in body weight and composition (Van Loan et al., 1998). Although a valid measure of BMD, dual-energy x-ray absorptiometry (DXA) may be limited in its focus to site-specific investigations, being limited to limbs rather than individual bones or bone regions. The value of BMD can also be limited by the inherent limitations of using a two-dimensional x-ray projection to

estimate bone area and geometrical changes (potential errors and the prevalence of errors with DXA are discussed in the literature (National Osteoporosis Society; Messina et al., 2015)). Heilmann et al. (1998) examined a number of imaging techniques in relation to forearm BMD and highlighted that all techniques have some degree of error. However, the use of peripheral quantitative tomography (pQCT) may be advantageous to examine these specific changes in future immobilisation studies.

It was also hypothesised that ω-3 would be the most effective supplement at minimising the effects of immobilisation. In fact, the data showed that neither ω-3 nor vitamin D had any significant effect on the responses to non-injurious immobilisation, other than on the associated accumulation of sub-cutaneous fatty tissue. Interestingly, it was observed that in the case of sub-cutaneous adiposity on the biceps brachii, there is a protective effect of both ω-3 supplementation against limb immobilisation. A few trends towards the attenuations in deleterious physiological events were observed in the ω-3 and vitamin D treated groups which were discussed within the chapter. Overall, however, at the current doses, ω-3 supplementation only significantly attenuated one of the changes (sub-cutaneous fatty tissue) associated with non-injurious limb immobilisation. These findings would necessitate further research into either a) supplementation linked to injury-induced immobilisation, or b) larger doses of these supplements to confirm/refute their physiological reserve preservation potential.

Chapter 4 focussed on ω-3 and vitamin D supplementation during immobilisation on the modulation of muscle functional, vascular and electromyographic profiles. As hypothesised, muscle elbow flexion and extension isometric and isokinetic torque decreased significantly with limb immobilisation. This is in line with the current literature that consistently demonstrates decreases

in strength with disuse models (Gondin et al., 2004; Hortobagyi et al., 2000; Miles et al., 1994; Yue et al., 1997). The decreases in maximal voluntary contraction (MVC) torque however, were not accompanied by any significant changes in biceps and triceps co-contraction RMS EMG values. EMG reliability is a general limitation of studies utilising longitudinal EMG monitoring (de Araujo et al., 2009; Fukuda et al., 2010). When drawing conclusions from EMG findings care should be taken, as: 1) the changes in muscle dimensions could result in a different population of motor units being recorded from (Clark and Fielding, 2012); 2) EMG data in the current studies, and previous work (Hermens et al., 2000), does not normalise the data for the clarity of the signal; 3) positioning of electrodes may differ slightly between repeated sessions. The changes in sub-cutaneous adiposity observed in Chapter 3 could have caused differences in the electromyographic signature (Solomonow et al., 1994). Similarly, the observed decrease in muscle thickness, arm girth and lean mass (i.e. dimensional changes) reported in Chapter 3 could result in a different population of motor units being recorded from (Clark and Fielding, 2012). It was hypothesised that vascular function would deteriorate with limb immobilisation. Previous research suggests that a decrease in physical activity leads to detrimental vascular adaptations (Delp et al., 2000; Louisy et al., 1997). The immobilisation model in this case had no impact on the assessed blood flow kinetics. Although resting measures of blood flow are a valid measure of vascular function/integrity, the use of reactive blood flow dimensions may be a better measure to detect the vascular adaptations with disuse.

As described in Chapter 1 there are equivocal findings of the impact of disuse models on muscle fatigability (Clark et al., 2008; Miles et al., 1994; Miles et al., 2005; Semmler et al., 2000). In Chapter 4, there was no significant change in muscle fatigue parameters with immobilisation. This supports the previous findings of Miles et al. (1994) who demonstrated that a shorter period of immobilisation in

the upper limb appeared to have minimal effects on muscle fatigability. However, other studies investigating the effects of immobilisation in the upper limb have found both increased (Clark et al., 2008; Semmler et al., 2000) and decreased (Miles et al., 2005) resistance to fatigue. The lack of changes in muscle fatigability in Chapter 3, however, goes hand in hand with the absence of vascularisation-related alterations (Sjøgaard et al., 1988; Enoka and Duchateau, 2008). The mechanisms that cause fatigue are specific to the task being performed (Enoka and Duchateau, 2008; Hunter et al., 2004). Therefore, variability between fatigue resistance responses to disuse models may be due to task specificity. The previous studies examining the response of muscle fatigability following arm immobilisation/suspension are of similar duration to the current chapter, ranging from three to four weeks. These studies all used various methods of assessing fatigability. They utilised time to task failure or repetition to task failure, at various intensities ranging from 15 to 50 % MVC. In comparison, in the current chapter, a maximum contraction was held for 30 seconds and fast Fourier transform (FFT) EMG response along with the mean, slope and Standard Deviation of the torque trace were utilised to assess muscle fatigability. The equivocal findings of the effects of disuse on muscle fatigability could be due to the duration of immobilisation or in the method used to test fatigue resistance. Studies investigating a comparison of different fatigue tasks before and after disuse are lacking.

As in Chapter 3, it was hypothesised again in Chapter 4 that ω-3 would be the most effective supplement at minimising the response to immobilisation. The current data demonstrate that neither ω-3 nor vitamin D had any significant effect on the responses to non-injurious immobilisation. Nonetheless, a few trends ($p<0.1$) towards the attenuation of elbow isometric and isokinetic torque immobilisation-induced decreases were observed, in the ω-3 and vitamin D treated

groups. Despite greater relative decreases in torque than in tissue composition (Chapter 3), there was no significant effect of ω-3 or vitamin D supplementation on the decreases in MVC torque. It would appear that muscle function might be a less reliable marker of the effectiveness of a supplement against the impact of immobilisation than tissue composition. This is particularly relevant, considering the protective effect of ω-3 in Chapter 3 against the decrease in adiposity.

Chapter 5 examined the systemic endocrine profile following 2 weeks of 9-waking-hours-per-day combined arm and shoulder immobilisation, with ω-3 and vitamin D supplementation. There were no significant changes in any of the analysed endocrine markers either at post-immobilisation or after re-mobilisation ($p>0.05$). Similarly, there was no supplement group effect on any of the above data ($p>0.05$). The current data, therefore, suggests that the degree and duration of immobilisation trialled in the present study, whilst capable of inducing muscle atrophy and declines in elbow torque, was not in fact a strong enough signal to elicit measurable changes at the systemic endocrine level. This suggests that the atrophy and strength declines seen in such a model (see Chapters 3 and 4) are not associated with changes in the specific enzyme and hormonal factors quantified here, at least not at the systemic level. These findings would be reassuring to any person suffering from a minor local joint sprain necessitating some restraint. In that, the immobilisation is unlikely to cause further deleterious physiological changes, distal to the initial site of immobilisation. There may however, be other endocrine factors, not monitored in this study, that may play crucial roles in skeletal muscle atrophy.

The final chapter (Chapter 6) investigated the most “promising” of the three supplements, which was determined to be EAA supplementation, in a pre-immobilisation supplementation model. The purpose of this study was to determine whether pre-immobilisation supplementation with an EAA supplement

before a period of limb immobilisation, would help to attenuate the declines in muscle morphology, muscle strength and bone parameters associated with upper limb immobilisation. As in the previous chapters, there was a significant change in muscle thickness, sub-cutaneous adipose thickness, arm girth, lean mass and isometric and isokinetic elbow flexion and extension torque following immobilisation. Also in line with the previous chapters, this immobilisation model had no impact on the assessed co-contraction and muscle fatigability, or on the assessed blood flow parameters. Despite significant immobilisation-induced changes in muscle size and strength and in adiposity, there was no significant impact of pre-immobilisation EAA supplementation. The idea behind this chapter was that building physiological reserve before immobilisation may better prepare the body for the up-coming immobilisation. In Chapter 6, there was a healthy, recreationally active group of participants, who reported adequate nutritional intake. It is still possible that in populations where malnutrition is present, that pre-loading, and therefore, increasing physiological reserve would be of benefit. Previous research has shown that pre-operative nutritional status can affect factors such as healing, rate of infection and length of hospital stay (Greene et al., 1991; Jevsevar and Karlin, 1993; Dempsey et al., 1988). Therefore, populations with malnutrition, who are known to be undergoing periods of disuse may benefit from nutritional supplementation before this event.

Throughout the data chapters and as discussed in Chapter 1 the decreases in muscle strength often surpass those seen in muscle size in disuse models (Berg et al., 1997; Clark et al., 2006; Kawakami et al., 2001; Miles et al., 1994; Narici et al., 1989; Seynnes et al., 2008; White et al., 1984). Therefore, other alterations in the neuromuscular system, other than the reduction in contractile proteins must contribute to the excessive loss of strength. Voluntary force production is associated with neurological and skeletal muscle properties, thus suggesting these

two factors as mechanisms accounting for the loss of strength with disuse models. The lack of changes in co-contraction EMG values in Chapter 4 and 6, suggests that factors other than muscle size and muscle co-activation alone must contribute to the excessive loss in muscle strength. These factors could include changes in connective tissue, muscle fibres, neurological innervation and endocrine status.

7.2. Implications

The overall aim of the current thesis was to determine the role that nutritional supplementation may play in attenuating hypo-activity-induced atrophy. Specifically, the objectives of the thesis were met in that the multi-parameter nature of the studies within this thesis allows for an in depth analysis of the numerous effects of immobilisation on skeletal muscle and the potential mechanisms underlying these changes. Many disuse studies only report one or two parameters and the current thesis adds to the literature, as it addresses multiple outcome parameters.

Nutritional supplementation may be an effective and easily adhered to intervention programme for preventing the loss of muscle mass/function seen with hypo-activity. The literature is lacking in immobilisation studies examining the impact of different nutritional supplements. The thesis introduces different supplements in the context of an upper limb immobilisation model. EAA proved the most “promising” of supplements and this adds to the body of research on proteins and essential amino acid supplements during disuse models. Despite the lack of effectiveness of ω-3 and vitamin D supplementation this is not a negative result. It simply means that at the current dose and in the specific population these supplements were not effective at reducing the detrimental effects of immobilisation. Pre-immobilisation supplementation with EAA did not attenuate the changes associated with immobilisation (Chapter 6), however, this should not be ruled out as a nutritional intervention in other disuse models or in sarcopenia for example. The “healthy” population examined in this study may not have benefited from this intervention but other demographics, e.g. an older population more likely to be nutrient deficient, may have done so.

Despite lower limb immobilisation resulting in greater atrophy than upper limb, it is just as important that we examine the effects of immobilisation in the arm as well as the leg. Injuries and surgery for example still occur in the upper limbs and therefore examination of how the body responds to the sub-sequent immobilisation is vital. The findings of the thesis are relevant to both sporting (e.g. off-season detraining modulation) as well as clinical (e.g. injury/illness induced short-term immobilisation/bed rest) populations. It is proposed that this relatively short-term sling immobilisation provides a model to be used to assess other supplements and treatments in future studies. A short-term model such as this is relatively less invasive and inconvenient for participants and may allow larger participant numbers to be recruited for research studies.

7.3. Limitations

7.3.1. Population

Despite participant numbers throughout the chapters being in-line with other disuse studies (Miles et al., 1994; Yue et al., 1997; Abe et al., 1997), the numbers are still relatively small. The participants used throughout this thesis were recreationally active and healthy young adults. As such these participants were not likely to exhibit an adverse nutritional intake background and would not be classed as an at risk group (NHS Livewell, 2010). The NHS consensus (2010) on vitamin D states that if people achieve a sufficient supply of vitamin D in the summer, most should keep levels greater than the deficiency threshold of 25 nmol/L in winter, even without supplementation.

7.3.2. Study design

All of the chapters used were randomised control trials, which are considered to be the gold standard for grouping participants in a study. The limitations of randomised control trials however, must be acknowledged, for example, the findings are protocol driven and the results are only specific to the population being examined.

7.3.3. Methodology

Despite the obvious benefits of a short-term immobilisation model and the observed detrimental effects of such a model, a more stringent long-term immobilisation or bed-rest model, may allow for the detection of a greater impact of nutritional supplementation. As discussed in Chapter 1 the most dramatic effects of disuse come from lower limb immobilisation studies. Therefore, the use

of such a model could reveal a more dramatic impact of immobilisation and therefore allowing for a greater attenuation with any nutritional supplementation.

Despite the multi-parameter nature of the studies throughout the thesis, some additional measures could have added to the findings. Chapter 2 data analysis was limited to a single site ultrasound scan, half way along the muscle length. In the proceeding chapters, multiple sites were then monitored and DXA analysis also added to measure total limb lean mass. As discussed above there is some degree of error with all imaging techniques and specifically DXA may be limited in its focus to site-specific investigations, being limited to limbs rather than individual bones or bone regions. The value of BMD can also be limited by the inherent limitations of using a two-dimensional x-ray projection to estimate bone area and geometrical changes. Therefore, the addition of pQCT throughout the thesis could have ruled out some of these factors. As discussed previously, resting measures of blood flow are a valid measure of vascular function/integrity. Despite this, the use of reactive blood flow dimensions may be a better measure to detect the vascular adaptations with disuse. The number of endocrine factors measured was limited to those reported in Chapter 6, this does not rule out changes in other endocrine factors during atrophy and that these factors were only measured at the systemic level.

7.3.4. Supplementation

In Chapter's 2 and 6 a multi-ingredient EAA supplement was provided. This multi-ingredient nature of the supplement makes it difficult to identify which specific amino acid or combination thereof, may have been the effective ingredient. The doses of ω-3 and vitamin D used in Chapter 3 to 5 were not sufficient to dramatically attenuate the detrimental effects of immobilisation. This could be because an over the counter dose is simply not enough to have significant effects

on such changes or simply that ω-3 and vitamin D are not suitable for attenuating changes in a seemingly ‘healthy’ population.

7.4. Directions for future research

The multi-parameter nature of the studies within this thesis allows for an in depth analysis of the numerous effects of disuse on skeletal muscle and the potential mechanisms underlying these changes. Future disuse studies should adopt the multi-parameter nature of this thesis, to increase the understanding of the complex processes that occur in response to disuse.

The relatively short-term sling immobilisation used throughout the thesis provides a model to be used to assess other supplements and treatments in future studies. A short-term model such as this is relatively less invasive and inconvenient for participants and may allow larger participant numbers to be recruited for research studies. Studies 3 and 4 consisted of eight participants in each supplement group. Participant numbers in disuse studies are notoriously small due to the inconvenient nature of models such as bed-rest and immobilisation (Miles et al., 1994; Yue et al., 1997; Abe et al., 1997). Ideally, research studies should look to recruit as many participants as possible and a short-term model may be a good way to achieve high participant numbers.

As discussed in the limitations section the participants used throughout this thesis were recreationally active and healthy young adults and as such may not have been likely to exhibit an adverse nutritional intake background and would not be classed as an at risk group. Future studies need to recruit those lacking in physiological reserve and those more at risk of malnutrition/deficiencies e.g. older and/or injured persons. Future research is warranted into the effects of nutritional supplementation with EAA, ω-3 and vitamin D in at risk groups before and/or during disuse models. The dosage of ω-3 and vitamin D may be addressed as at the current dosage only one parameter had a significant attenuation with ω-3 or supplementation. Despite the obvious benefits of the short-term immobilisation

model, a more stringent long-term immobilisation or bed-rest model, may allow for the detection of a greater impact of nutritional supplementation.

Ultrasound and DXA were highly reliable techniques used throughout this thesis that detected significant changes in muscle thickness, sub-cutaneous adipose thickness and lean mass. Site-specific changes in sub-cutaneous adipose thickness were detected with no significant change in DXA quantified fat mass and percentage (Chapter 3). The use of magnetic resonance imaging (MRI) in future studies would allow for multi-site measurements along a limb to determine exactly where changes are occurring. As mentioned earlier in the discussion, BMD and BMC values can be influenced by changes in body weight and composition and can be limited by the inherent limitations of using a two-dimensional x-ray projection to estimate bone area and geometrical changes. Future studies could benefit from the addition of the use of pQCT to examine specific changes.

Studies investigating a comparison of different fatigue tasks before and after periods of disuse are sparse. More research is needed to investigate the task-specific responses to different models of disuse and the potential mechanisms behind the differential responses. The lack of changes in muscle fatigability and resting arterial blood flow in Chapters 4 and 6 go hand in hand. Future studies should examine reactive blood flow rather than resting to observe any changes that could then be associated with fatigability.

The ideal treatment to help prevent declines in muscle size and strength with disuse would be exercise prescription with adequate nutritional intake. As stated previously exercise prescription is not always a practical prescription, and therefore, other interventions are needed. Potentially, nutrition alone will not be able to fully prevent the deleterious effects of disuse but if it is able to attenuate such changes then this is still of benefit. In particular, it may be that the “at risk”

groups discussed previously (i.e. aged populations) would benefit more so from nutritional interventions to reach adequate dietary intake.

7.3. Conclusions

The main findings of this thesis demonstrate that a relatively short-term arm immobilisation results in a measurable decrease in muscle thickness, arm girth, lean mass, isometric elbow torque, isokinetic elbow torque and an increase in sub-cutaneous adipose thickness. Muscle fatigability, resting arterial blood flow (heart rate, resistance index and flow by diameter), EMG co-contraction and endocrine markers however are unchanged following this arm immobilisation model. The sling immobilisation used is proposed as a suitable model for observing the effects of relatively short-term immobilisation.

At the current dosage ω-3 supplementation only attenuated the increase in sub-cutaneous adipose thickness in the biceps brachii. Despite some trends, neither ω-3 nor vitamin D supplementation attenuated any other parameters that were changed during immobilisation. These findings would necessitate further research into either: a) supplementation linked to injury-induced immobilisation, or b) larger doses of these supplements to confirm/refute the physiological reserve potential of the two supplements.

EAA supplementation during the three weeks of immobilisation impacted positively on the immobilisation-induced changes in the structural and functional characteristic of the remaining muscle. EAA supplementation was highlighted as the “most promising” nutritional supplement and as such was chosen for Chapter 6 and provided as a pre-immobilisation supplement. Providing EAA supplementation before immobilisation did not attenuate the immobilisation-induced changes in muscle structure and function. It is suggested that EAA supplementation pre-immobilisation in a healthy population in which physiological reserve is satisfactory may not be beneficial. Future studies are warranted in more “at risk” populations, e.g. older populations and people with certain musculoskeletal wasting conditions.

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Appendices

Appendix 1: Publications

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REVIEW

Hypo-activity induced skeletal muscle atrophy and potential nutritional interventions: A review

Emma L Bostock, Christopher I Morse, Keith Winwood, Islay McEwan, Gladys L Onambélé-Pearson

Emma L Bostock, Christopher I Morse, Keith Winwood, Islay McEwan, Gladys L Onambélé-Pearson, Institute for Performance Research, Department of Exercise and Sport Science, Manchester Metropolitan University, Crewe CW1 5DU, United Kingdom

Author contributions: All the authors contributed to this work.

Correspondence to: Gladys L Onambélé-Pearson, PhD, Institute for Performance Research, Department of Exercise and Sport Science, Manchester Metropolitan University, Crewe Green Road, Valentine Building, Room 2-7, Crewe CW1 5DU, United Kingdom. g.pearson@mmu.ac.uk

Telephone: +44-161-2475594 Fax: +44-161-2476386

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Abstract

Periods of hypo-activity result in profound changes in skeletal muscle morphology and strength. This review primarily addresses the differential impact of de-training, bed-rest, limb immobilisation and unilateral lower limb suspension on muscle morphology, strength and fatigability. The degree of muscle atrophy differs depending on the hypo-activity model and the muscles in question, with the leg and postural muscles being the most susceptible to atrophy. Hypo-activity also results in the dramatic loss of strength that often surpasses the loss of muscle mass, and consequently, the nervous system and contractile properties adapt to adjust for this excessive loss of strength. In addition, the degree of muscle strength loss is different depending on the hypo-activity model, with immobilisation appearing to have a greater impact on strength than unloaded models. There is a step-wise difference in the magnitude of muscle loss so that, even after accounting for differential durations of interventions immobilisation ⩾ unilateral lower limb suspension ⩾ bed-rest ⩾ de-training. Muscle fatigability varies between hypo-activity models but the results are equivocal and this may be due to task-specific adaptations. This review also addresses potential nutritional interventions for attenuating hypo-activity induced muscle atrophy and strength declines, in the absence of exercise. Essential amino acid supplementation stands as a strong candidate but other supplements are good contenders for attenuating hypo-activity induced atrophy and strength losses. Several potential nutritional supplements are highlighted that could be used to combat muscle atrophy but extensive research is needed to determine the most effective.

Key words: Immobilisation; Disuse; Muscle size; Muscle strength; Nutrition supplementation; Muscle fatigability

Core tip: This review summarises and compares the morphological, strength and fatigability changes in response to different models of hypo-activity. The hypo-activity models include de-training, bed-rest, immobilisation and unilateral lower limb suspension. There is a step-wise difference in the magnitude of muscle and somewhat strength losses so that, even after accounting for differential durations of interventions immobilisation ⩾ unilateral lower limb suspension ⩾ bed-rest ⩾ de-training. Muscle fatigability varies between hypo-activity models but the results are equivocal and this may be due to task-specific adaptations. This review also highlights several potential nutritional interventions for attenuating hypo-activity induced changes.

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INTRODUCTION

Skeletal muscle is one of the most adaptable tissues in the body, and as such, it is capable of altering its structure in response to different levels of physical activity. Prolonged reductions in muscle activity and mechanical loading result in many physiological adaptations in skeletal muscle form and function[1-4]. Muscle atrophy (decrease in muscle mass) is seen during reduced activity (*e.g.*, sedentary behaviour, de-training)[5-8] or disuse models (*e.g.*, immobilisation, head-down tilt bed-rest)[1,3,9,10]. It is evident that the degree of muscle atrophy is not constant across muscle groups or hypo-activity models[2,3,11,12].

Simply reducing normal levels of activity can be classed as the first stage of disuse. Decrements in muscle mass and strength have been documented in trained humans undergoing de-training[5-8,13-15]. Bed-rest conditions result in the removal of normal weight-bearing forces acting on the bones of the lower limbs in the vertical position and a decrease in number and/or magnitude of muscle contractions, particularly in the postural musculature. During bed-rest, muscular contraction is still possible although it is limited and the muscular force required for producing movement is very much diminished once ground reaction forces are removed. A more rigid immobilisation can be achieved by casting a limb, resulting in more rapid decrements in muscle mass than does bed-rest alone. The final method of hypo-activity commonly reported in the literature is that of unilateral lower limb suspension (ULLS), a method of reducing habitual activity whilst causing lesser degree of inconvenience to the participants.

The purpose of this review is to assess the varying impact of different hypo-activity models on the skeletal muscle system. This is broken down into the effects of hypo-activity on muscle morphology, muscle strength and muscle fatigability. In order to provide some homogeneity in the results based on the variable duration of the hypo-activity, values are presented per week and where relevant the duration of the hypo-activity is provided in parenthesis. Exercise prescription is not always a practical prescription, even when it would be recommendable to individuals under-going immobilisation or bed-rest after trauma or illness, due to the presence of counter indications for exercise such as pain, immobilisation in a cast, *etc.* Thus, other interventions are required to attenuate losses in muscle mass and function. Therefore, this review will also discuss potential nutritional interventions for preventing the loss of muscle mass/function seen with hypo-activity, where increased physical activity is not combined with the nutritional treatment. Studies were found using search terms "bed-rest and atrophy" and "immobilisation and atrophy" in PubMed. However, this returned over 1400 hits. To focus our search criteria, only data on healthy humans were selected through the inclusion of the "human" and "clinical trial" filters in the PubMed search. This resulted in 86 studies, suitable for inclusion in the present review.

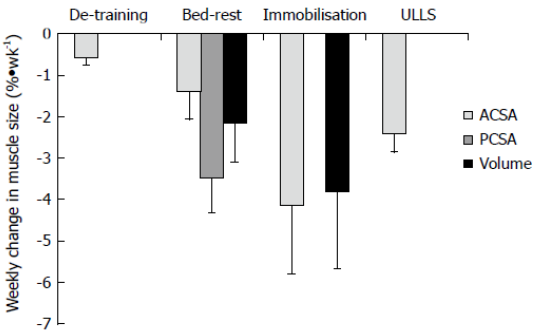

Figure 1 Relative change in muscle anatomical cross sectional area, physiological cross sectional area and volume. ULLS: Unilateral lower limb suspension; ACSA: Anatomical cross sectional area; PCSA: Physiological cross sectional area.

MUSCLE MORPHOLOGY

Muscle anatomical cross sectional area

Anatomical cross sectional area (ACSA) is the cross-sectional area of the muscle at right angles to its longitudinal axis. Muscle ACSA is a major determinant of maximum voluntary contraction (MVC) torque[16,17] and hypo-activity models have been shown to result in the decrease in this parameter. [Figure 1 shows relative change in muscle anatomical cross sectional area (ACSA), physiological cross sectional area (PCSA) and volume in response to hypo-activity models. Values are taken from the references used in the text for de-training (ACSA-40 d and 24 wk)[6,8], bed-rest (ACSA-30 d to 17 wk)[2,11,18,19] (PCSA-20 d)[1,9,20] (Volume-7 d and 32 d)[11,21], immobilisation (ACSA-9 d to 4 wk)[3,4,12,22-26] (Volume-2 wk and 4 wk)[10,12,27] and ULLS (ACSA-23 d and 4 wk)[28-31]. Where there are missing bars, this shows gaps in the literature (*i.e.*, values are not available for a parameter during a specific hypo-activity model). Values are presented as means; error bars denote SD]. Periods of detraining (24 wk) have resulted in a decrease in ACSA of the quadriceps[6]. Likewise, Narici *et al*[8] reported decreases in leg ACSA (approximately 0.7%/wk) in response to 40 d de-training.

Stricter hypo-activity models result in greater decreases in muscle ACSA. Following 30 d bed-rest Convertino *et al*[11] reported decreases in ACSA of the calf (approximately 1.1%/wk) and thigh (approximately 1.9%/wk). Similarly, a 2.4%/wk decrease in plantar flexors was found following 5 wk horizontal bed-rest[18]. Muscle group-specific adaptations have been demonstrated in skeletal muscle ACSA of the leg and lumbar musculature after 17 wk of bed-rest[2]. The plantar-flexors were more susceptible to atrophy (approximately 1.8%/wk) than the dorsiflexors (approximately 0.9% to 1.2%/wk)[2]. The intrinsic lumbar muscles atrophied approximately 0.5%/wk but there was no significant change in psoas muscle mass[2]. Rittweger *et al*[19] reported a decrease in calf muscle ACSA (approximately 2.0%/wk), which was greater than the reported decrease in the forearm ACSA (0.5%/wk) in response to 90 d bed-rest.

Immobilisation of the leg through plaster cast has shown to decrease calf ACSA (approximately 3% to 5%/wk) after just 2 wk[4,22]. Changes in quadriceps ACSA (approximately 8.3%/wk) have also been documented with as little as 10 d leg cast immobilisation[25]. Similarly, Veldhuizen *et al*[3] reported decreases in quadriceps ACSA (approximately 5.3%/wk) with 4 wk leg casting. Immobilisation of the knee using a brace has also resulted in decreases in muscle ACSA[24,26]. Fourteen days of knee-brace immobilisation has resulted in ACSA decreases of the thigh (approximately 3.1%/wk), quadriceps (approximately 2.9% to 3.8%/wk), gastrocnemius (approximately 4.7%/wk) and soleus (approximately 3.3%/wk) muscles[24,26]. Yasuda *et al*[26] found no sex-based differences in the quadriceps ACSA response to knee-brace mediated immobilisation. There is considerably less data on immobilisation-induced atrophy of the upper limb muscles. Casting of the arm for as little as 9 d has shown to decrease ACSA of the forearm (approximately 3.2%/wk)[23]. Yue *et al*[12] investigated the effect of 4 wk elbow joint immobilisation with a fibre glass cast and reported a decrease in elbow flexor ACSA (approximately 2.8%/wk).

Tesch *et al*[32] developed a model to study the effects of an unloaded limb in humans that allows for freely moveable joints but minimises load bearing. In this ULLS method, a sling suspends one lower leg and the contralateral shoe has an elevated sole to allow for a relaxed position of the unloaded limb. ULLS also results in decreases in muscle ACSA, though to a lesser degree than immobilisation. ULLS of 23 d has been reported to decrease knee extensor (approximately 3%/wk)[30] and plantar flexor (approximately 2.7%/wk)[31] ACSA. Correspondingly, Clark *et al*[28,29] reported decreases in plantar flexor (approximately 2.0% to 2.3%/wk) and knee extensor (approximately 2.0%/wk)[29] ACSA in response to 4 wk ULLS. It would therefore seem that in terms of ACSA at least, the most impactful model of hypo-activity is immobilisation.

Muscle physiological cross sectional area

PCSA is the area of the muscle at right angles to the longitudinal axis of the fibres. Muscle PCSA has been associated with the maximal force generating capacity of a muscle[33] and has been shown to decrease with bed-rest[1,9,20]. Twenty days bed-rest has been shown to decrease PCSA of the thigh (between approximately 2.7% to 3.6%/wk)[1,20]. Akima *et al*[9] described muscle group-specific adaptations, demonstrating a decrease in PCSA of knee extensor (approximately 2.5%/wk), knee flexor (approximately 4.0%/wk) and plantarflexor (approximately 4.5%/wk) muscles in response to 20 d of 6 degrees head-down-tilt bed rest. It is generally accepted that muscle losses are greater in the knee extensors than the knee flexors after unloading in humans[34]. Akima *et al*[9] demonstrated the opposite to this, which could be due to the methodology used to determine PCSA. In addition, since a muscle placed in a shortened position experiences a greater degree of atrophy than one placed in a lengthened position[35], the pattern/magnitude of disuse would therefore be expected to be modulated by both the mode of hypo-activity and the joint angle adopted in the immobilisation. Bed-rest, however, had no effect on the PCSA of the tibialis anterior[9]. The tibialis anterior experiences lower activation during habitual physical activities than other muscles such as the plantar flexor, and as such may explain the lack of decrease in tibialis anterior muscle PCSA with bed-rest. Comparisons of bed-rest to other hypo-activity models in terms of PCSA changes is not yet possible, as research is lacking with this parameter being measured.

Muscle volume

Muscle volume is a major determinant of joint torque[36] and has been shown to decrease in response to bed-rest and immobilisation models[10-12,21,27]. Muscle volume of the thigh decreases (approximately 3%/wk) with as little as 7 d bed rest[21]. Following 30 d bed rest, Convertino *et al*[11] reported decreases in calculated leg volumes of the calf (approximately 2.3%/wk) and thigh (approximately 1.1%/wk). Yue *et al*[12] investigated the effect of 4 wk elbow joint immobilisation with a fibre glass cast and reported a decrease in elbow flexor volume (approximately 2.9%/wk). A case study of a orthopaedic patient who fractured the fifth metatarsal of the right foot displayed substantial and rapid losses in muscle volume, both proximally and distally to the immobilisation site after 4 wk subsequent immobilisation[10]. The degree of muscle volume decrease varied between the different muscle sites of the triceps surae (approximately 5.5%/wk), quadriceps (approximately 6.0%/wk) and hamstrings (approximately 1.6%/wk)[10]. This is in agreement with the general acceptance that muscle volume is lost to a greater extent in the knee extensors compared to the knee flexors[34]. An age-related susceptibility to immobilization i also evident whereby, Urso *et al*[27] demonstrated different responses to 2 wk adductor pollicis (AP) immobilisation between younger and older males. AP volume decreased approximately 2.1%/wk (not significant) in young males and significantly decreased by approximately 4.8%/wk in older males[27].

Upper vs lower limb

Immobilisation through casting appears to have a greater effect on the lower limb musculature than the upper body. This is not surprising since the habitual loading of the lower extremities, because of body weight in normal ambulation and even in the absence of intended physical exertion, is far more substantial than that in the upper extremities. Understandably, this thereby affects the required threshold of decrease in muscle activity necessary to negatively impact on muscle metabolism. [Relative change in muscle ACSA, PCSA and volume in response to hypo-activity models. Values are separated into the effect of each hypo-activity model on the upper limb (UL) *vs* the lower limb (LL). The values are taken from the refer-

Table 1 Relative change in upper and lower limb muscle anatomical cross sectional area, physiological cross sectional area and volume

	ACSA_UL (%)	ACSA_LL (%)	PCSA_UL (%)	PCSA_LL (%)	Volume_UL(%)	Volume_LL (%)
De-training	-	-0.6	-	-	-	-
Bed-rest	-0.5	-1.5	-	-3.5	-	-2.1
Immobilisation	-3	-4.4	-	-	-3.3	-4.4
ULLS	-	-2.4	-	-	-	-
Mean (SD) of 4 models	-1.8 (1.8)	-2.2 (1.6)		-3.5 (0.01)	-3.3 (0.01)	-3.3 (1.6)

ACSA: Anatomical cross sectional area; PCSA: Physiological cross sectional area; ULLS: Unilateral lower limb suspension; UL: Upper limb; LL: Lower limb.

ences used in the text for de-training (ACSA_LL)[6,8], bed-rest (ACSA_UL)[19] (ACSA_LL)[2,11,18,19] (PCSA_LL)[1,9,20] (Volume_LL)[11,21], immobilisation (ACSA_UL)[12,23] (ACSA_LL)[3,4,22,24-26] (Volume_UL)[12,27] (Volume_LL)[32] and ULLS (ACSA_LL)[28-31]. Where there are missing values, this shows gaps in the literature *(i.e.*, values are not available for a parameter during a specific hypo-activity model) (Table 1)]. Forearm muscle ACSA decreased 4.1% with 9 d arm casting[23], whereas, a similar period of immobilisation of the lower limb with 10 d casting resulted in an 11.8% decrease in quadriceps ACSA[25]. Similarly, with longer periods of immobilisation the effect seems to be greater in the lower limbs. In response to 4 wk elbow joint casting, Yue *et al*[12] reported an 11.2% decrease in elbow flexor ACSA, whereas, Veldhuizen *et al*[3] reported a 21% decrease in quadriceps ACSA in response to 4 wk leg casting.

Intramuscular adipose tissue

Using signal intensity analysis of lower limb magnetic resonance images (MRI). Manini *et al*[37] discriminated between the relative changes in adipose and skeletal muscle tissue resulting from a 4 wk period of ULLS. In addition to the characteristic reduction in muscle ACSA, there was a concomitant 15% increase in intermuscular adipose content after 4 wk of lower limb suspension[37]. Thus, these findings suggest, that hypo-activity induced alterations in skeletal muscle morphology goes beyond muscle atrophy alone.

Summary

Together, these findings show that the extent of muscle atrophy differs depending on the hypo-activity model. Certain factors may modulate the differential responses to hypo-activity models (*e.g.*, age, nutritional status). Indeed, both Kortebein *et al*[38] and Urso *et al*[27] suggested that older individuals experience greater losses in muscle mass when compared to younger individuals. A change in nutritional status, whether it is due to physiological changes directly caused by hypo-activity or to altered behaviour that is caused by hypo-activity and leads to changes in diet, could affect the physiological systems in question. The above also suggest that the degree of muscle atrophy differs between muscle groups, with the leg and postural muscles being most susceptible to atrophy. This is likely to be due to the comparatively substantial decrease in habitual weight-bearing forces applied to the lower limb during hypo-activity. Hypo-activity not only decreases muscle content, but also impacts on the intrinsic composition of the said skeletal muscle through increased adiposity[37] and altered muscle architecture[39].

The decrease in muscle mass seen with hypo-activity may be the result of an imbalance between protein synthesis and protein breakdown[40-42]. In response to 14 d simulated microgravity, Ferrando *et al*[40] reported a loss of lean muscle mass, accompanied with a 14% decrease in protein synthesis and no change in protein breakdown. Similarly, Gibson *et al*[41] reported a marked fall in muscle protein synthesis in response to 7 wk leg immobilisation. A shorter period of immobilisation (21 d) provided little evidence of increases in mRNA for catabolic enzymes or increases in enzyme activity during this period[43]. However, there is some evidence to suggest that increases in catabolic potential do occur, and that this event happens very quickly (48 h) after immobilisation[42]. Nevertheless, collectively the evidence suggests that protein breakdown is unlikely to be a key modulator in the process of muscle atrophy occurring during immobilisation in humans[44,45].

The molecular signalling responses to de-training are only just beginning to be investigated, and to date, only changes in metabolic proteins have been reported in human skeletal muscle[46,47]. With bed-rest, Ogawa *et al*[48] reported increased mRNA expression of the E3 ligases, Cbl-b and Atrogin-1 in response to 20 d bed-rest. This was accompanied by a threefold increase in ubiquitinated proteins[48]. Investigation into the effects of limb immobilisation on cell signalling in humans is limited. Modest changes in mRNA for many genes in the first 2 d after immobilisation have been reported but these changes do not affect protein levels of most transcripts[42]. However, the Akt protein synthesis pathway and extracellular matrix components seem to be affected within 48 hours of immobilisation[42]. Chen *et al*[49] and Jones *et al*[50] reported increases in the E3 ligases, Atrogin-1 and MuRF-1 in response to 11 to 14 d immobilisation in humans. These changes were not seen with 48 h immobilisation[42] and are therefore thought to only occur after long duration (days rather than hours) immobilisation. Increased metallothionein expression in human skeletal muscle fibres has been associated with exposure to physiological stress, which results in elevated levels of reactive oxygen species

(ROS)[51]. Urso *et al*[42] reported a more than two-fold increase in metallothioneins in human skeletal muscle with 48 h of immobilisation. However, neither Chen *et al*[49] nor Jones *et al*[50] identified changes with longer periods of immobilisation. This may suggest that metallothioneins are increased in the first few days of hypo-activity to prevent ROS-mediated DNA or cellular damage. de Boer *et al*[43] investigated the effects of ULLS on gene expression and cell signalling. They reported increased expression of mRNA for MuRF-1 by approximately 3 fold after 10 d without changes in MAFbx or tripeptidyl peptidase II mRNA, but all decreased between 10 and 21 d[43]. These authors concluded that both myofibrillar and tendon protein synthetic rates show progressive decreases during 21 d of disuse; in muscle this is accompanied by decreased phosphorylation of FAK, with no marked increases in genes for proteolytic enzymes[43]. Overall, whilst it is clear that cell signalling responses differ between hypo-activity models; further research is needed to provide a definitive description of the timing, magnitude and nature of these molecular adaptations.

MUSCLE STRENGTH

The associated decline in strength through hypo-activity can be best described based on the mode of assessment. Both isometric and dynamic strength have been reported to decline with hypo-activity, the relative magnitude of which appears to largely reflect the patterns of atrophy described above.

Isometric strength

Hypo-activity models alter muscular isometric torque. After 40 d de-training, Narici *et al*[5] reported a decrease in knee extension isometric MVC (approximately 2.1%/wk). Similarly, maximum isometric quadriceps strength has been reported to decrease with 90 d de-training (approximately 1.3%/wk)[5]. More dramatic losses in isometric torque are seen with stricter hypo-activity models. Bed-rest models have been shown to decrease maximum voluntary force of plantar flexion (approximately 7.5%/wk)[52] and knee extensor torque (approximately 4.1% to 5.0%/wk)[53]. Correspondingly, Kawakami *et al*[1] showed a decrease in muscle force for knee extension (approximately 3.8%/wk) with 20 d bed-rest.

Studies using 2 wk of cast immobilisation have reported decreases in triceps surae isometric MVC torque (approximately 8.5 and 12%/wk)[4,54]. A discrepancy between the two studies may be due to the degree of immobilisation. Gondin *et al*[54] simply immobilised the ankle joint, whilst, White *et al*[4] utilised a full leg cast. Knee-brace mediated immobilisation has resulted in a decrease in knee extensor and plantar flexion isometric strength (approximately 11.2 and 12.7%/wk, respectively)[24]. Knee-cast mediated immobilisation resulted in a slightly larger decrease in isometric leg strength (approximately 15.7%/wk)[55]. Christensen *et al*[22] utilised a knee-to-toe plaster cast and reported a decrease in isometric calf muscle strength (approximately 4.5%/wk). Studies using casting to immobilise the elbow joint have found decreases in isometric MVC of the elbow flexors (approximately 5.3% to 8.8%/wk)[12,56,57], and a decrease in the maximum load that could be lifted[12]. A more dramatic decrease in isometric MVC torque has been reported in the flexors and extensors of the wrist (approximately 22.8% to 25.3%/wk) in response to immobilisation[23,58].

With ULLS, isometric torque appears to be affected to a lesser degree than with immobilisation models. An explanation for the above observation may be that ULLS removes weight-bearing but allows for freely moveable joints (hence a degree of muscular activity) whereas immobilisation is a more rigid model that does not allow joint movement (hence a greater restriction of muscular activity). Studies have reported plantar flexor isometric MVC torque to decrease (approximately 5% to 7 %/wk) with ULLS[28,31]. With ULLS, increased fluctuations in plantar flexion (approximately 3%/wk) and knee extension (approximately 5.5%/wk) isometric force have been demonstrated[29].

Isokinetic strength

In addition to the established decline in isometric strength (torque and force), hypo-activity models (de-training, bed-rest, immobilisation and ULLS) also result in reductions in dynamic torque outputs. Hypo-activity models also result in changes to dynamic torque outputs. After 14 d de-training isokinetic eccentric and concentric knee extension force has been shown to decrease by approximately 6% and 1.2%/wk, respectively[7]. With as little as 14 d bed-rest decrements in knee extensor 1 repetition maximum (approximately 4.5%/wk) are seen along with a fall in MVC (approximately 7.5%/wk)[59]. After 6 wk bed-rest maximum voluntary concentric knee extensor torque was shown to decrease uniformly across angular velocities (approximately 4.1% to 5.0%/wk)[53]. Muscle-specific adaptations are evident with bed-rest, as shown by Dudley *et al*[60] who reported a decrease in concentric and eccentric isokinetic knee extensor peak torque (approximately 4.4%/wk), with no alterations in knee flexors in response to 30 d 6 degrees head-down bed-rest. Again muscle-specific adaptations were demonstrated by LeBlanc *et al*[18] who reported a decrease in plantar flexor concentric isokinetic strength (approximately 2.6%/wk) and no change in the isokinetic strength of the dorsiflexors with 5 wk bed-rest. As with the knee extensors *vs* knee flexors difference in sensitivity to hypo-activity alluded to above, the plantar flexor muscles experience a greater level of recruitment during gait than the tibialis anterior. Thus, habitual muscle recruitment prior to hypo-activity would appear to be a large determinant of the relative magnitude of hypo-activity-induced changes.

Results from lower limb immobilisation models indicate that short-term immobilisation is associated not only with atrophy but with a diminished capacity of the muscle to perform both concentric and eccentric strength[23,55]. Lower limb casting results in a dramatic

decrease in isokinetic quadriceps strength (approximately 29.1%/wk)[25]. There is evidence that the effect of leg cast immobilisation on isokinetic strength of the knee extensors and flexors is greater in the knee extensors, demonstrated by a fall in peak torque of approximately 13.3%/wk for the knee extensors and approximately 3.3%/wk for knee flexors[3]. Cast immobilisation of the arm also results in decreased concentric (approximately 6.9% to 16.9%/wk) and eccentric (approximately 9.7% to 14.4%/wk) strength for flexion, extension, pronation and supination of the wrist[23].

Less dramatic decreases in isokinetic strength are seen with ULLS compared to immobilisation. de Boer *et al*[30] found a decrease in isokinetic knee extensor torque in response to 23 d ULLS (approximately 6.4%/wk). Similarly, after 4 wk ULLS mean average peak isokinetic torque is decreased (approximately 4.3%/wk)[61]. With as little as 14 d ULLS, a decrease in peak isokinetic torque (approximately 5% to 8.6%/wk) and total work performed (approximately 7.5% to 10.0%/wk) by knee extensors and flexors was reported[62].

Strength vs size changes

There is evidence to suggest that decreases seen in strength in response to hypo-activity models are greater than the changes seen in muscle size. With de-training the loss in leg muscle ACSA (approximately 0.7%/wk) was not as great as the decrease seen in knee extension MVC (approximately 2.1%/wk)[8]. Similarly, in bed-rest Kawakami *et al*[1] suggested that the decrease in knee extension mean muscle force (approximately 3.8%/wk) seen after 20 d head down bed-rest was related more to changes in neural activation to those in PCSA (approximately 2.7%/wk). Correspondingly, Berg *et al*[53] suggested that the decline seen in strength (approximately 4.1% to 5.0%/wk) could not be entirely accounted for by decreased ACSA (approximately 2.3%/wk), and that the strength loss could also be due to factors resulting in decreased neural input to muscle and/or reduced specific tension of muscle, as evidenced by a decreased torque to EMG ratio. Discrepancies between decreases in muscle size and muscle strength have also been reported in upper and lower immobilisation studies. White *et al*[4] reported an approximately 5%/wk decrease in muscle ACSA whilst triceps surae MVC decreased approximately 12%/wk. Additionally, the upper limb decreases in forearm ACSA (approximately 3.2%/wk) were much smaller than those reported in forearm flexor and extensor strength (approximately 22.8% to 25.3 %/wk)[23]. Again, in ULLS models muscle torque (approximately 5% to 7%/wk) appears to decrease to a greater degree than muscle ACSA (approximately 2.3% to 2.7%/wk)[28,31].

Summary

Bed-rest appears to have varying degrees of impact on the upper and lower body. After 14 d of 6 degrees head down bed-rest maximum voluntary force for plantar flexion was decreased (approximately 7.5%/wk) whilst no effect was observed on maximal voluntary force of

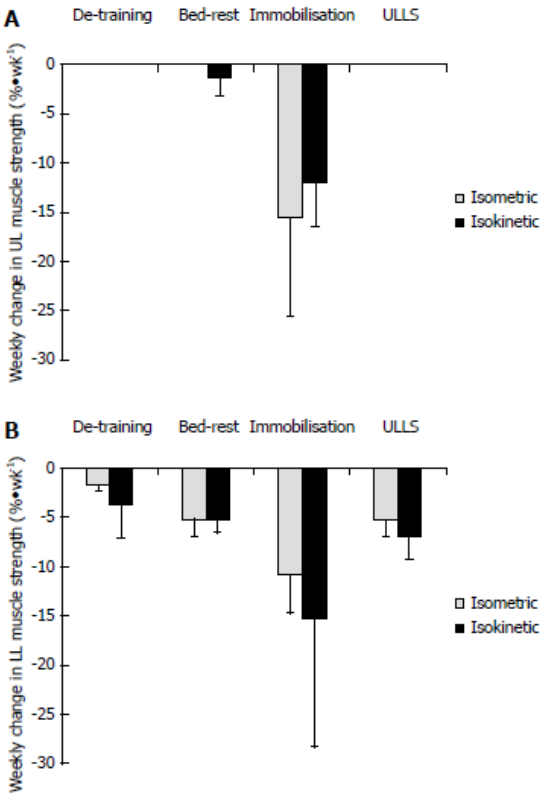

Figure 2 Relative change in isometric and isokinetic strength. A: Upper limb; B: Lower limb. ULLS: Unilateral lower limb suspension; UL: Upper limb; LL: Lower limb.

hand grip[52]. Similar results were demonstrated by LeBlanc *et al*[2] who showed after 17 wk of continuous bed-rest that isokinetic muscle strength decreased significantly in the thigh and calf with no loss in the arms. These results further support the idea that the lower limbs are primarily affected by bed-rest, more so than the upper limb. However, Gogia *et al*[63] did observe a decrease in elbow flexor torque (approximately 3.8%/wk) and a non-significant decrease in elbow extension torque (approximately 1.4%/wk) after 5 wk of bed-rest. Thus, suggesting that strength in the upper limb is affected by bed-rest but only in specific muscles during specific tasks.

Together, these findings show that in addition to the reduction in muscle mass, hypo-activity also results in a dramatic loss of strength [Figure 2 relative change in isometric and isokinetic strength in response to hypo-activity models. Figure 2A Values taken from references in the text for upper body changes in strength in response to de-training, bed-rest (isokinetic)[2,52,63], immobilisation (isometric)[12,23,56-58] (isokinetic)[23] and ULLS. Figure 2B values taken from references in the text for lower body changes in strength in response to de-training (isometric)[5,8] (isokinetic)[7], bed-rest (isometric)[1,52,53] (isokinetic)[18,53,59,60], immobilisation (isometric)[4,22,24,54,55] (isokinetic)[3,25] and ULLS (isometric)[28,29,31] (isokinetic)[30,61,62]. Where there are missing bars, this shows gaps in the

literature (*i.e.*, values are not available for that parameter during a specific hypo-activity models). Values are presented as means; error bars denote SD]. Models in which the joint is immobilised appear to have a greater impact on strength than unloaded models. These changes in muscular strength vary between hypo-activity models. The degree of loss in muscular strength surpasses the loss of muscle mass. Therefore, other alterations in the neuromuscular system, other than the reduction in contractile proteins must contribute to the excessive loss of strength. Voluntary force production is associated with neurological and skeletal muscle properties, thus suggesting these two factors as mechanisms accounting for the loss of strength with hypo-activity models.

Muscle fatigability

Studies have also examined the impact of hypo-activity models on the fatigability of skeletal muscle. Kamiya *et al*[64] showed no change in time to fatigue after 14 d bed-rest. After a longer period of bed-rest (8 wk), Mulder *et al*[65] demonstrated an increase in fatigability (7.2%-10.2%/min decrease in maximum voluntary isometric torque per minute exercise; or approximately 0.9%-1.3%/wk fatigability increment). The contrast between the two studies would tend to suggest a delay in the impact of hypo-activity on muscle fatigability.

The effect of immobilising a limb has various different effects on skeletal muscle fatigability. Two weeks of full leg cast immobilisation resulted in no effect on muscle fatigability[4]. In contrast, Veldhuizen *et al*[3] found a decrease in isokinetic quadriceps endurance work from 9.1 kJ to 5.6 kJ after 4 wk leg cast immobilisation. These results suggest that short periods of lower limb immobilisation ($\leqslant$ 2 wk) have little effect on muscle fatigability whilst longer periods of immobilisation ($\geqslant$ 4 wk) increases muscle fatigability. Studies investigating the effects of immobilisation on skeletal muscle fatigability in the upper limbs have found different effects to those in the lower limbs. Similar to lower limbs shorter periods of immobilisation in the upper limbs appear to have minimal effects on muscle fatigability[23]. Unlike the lower limb, longer periods of immobilisation of the upper limb show a trend towards increased resistance to fatigability. Following 3 wk of hand-forearm immobilisation time to task failure increased by 21% (approximately 7%/wk)[66]. Semmler *et al*[56] investigated the effects of fiberglass cast immobilisation of the elbow joint, and reported 7 out of the 12 immobilised participants exhibited an unusual pattern of muscle activity during a fatiguing contraction after immobilisation. In those individuals with this unusual pattern of muscle activity there was an associated increase in the ability to maintain a contraction over an extended period of time in the elbow flexor muscles[56]. The physiological basis for the sometimes observed immobilisation-induced decreased fatigability, is not clear but it is likely to be related to neural factors[56]. In contrast to this, Miles *et al*[67] found an increase in fatigability in response to 3 wk arm suspension in untrained but not trained individuals. Previous research showed that ULLS led to increased fatigability after 4 wk of unloading[61]. Results from Deschenes *et al*[62] found a contrasting decrease in fatigability after just 2 wk of unloading.

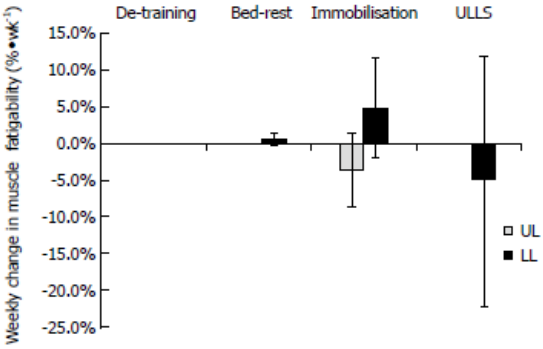

Figure 3 Relative change in muscle fatigability. ULLS: Unilateral lower limb suspension; UL: Upper limb; LL: Lower limb.

Collectively these results suggest that muscle fatigability varies between different hypo-activity models [Figure 3 relative change in muscle fatigability in response to hypo-activity models (mean ± SD). Positive percentage change depicts an increase in fatigability whilst negative percentage change shows a decrease in fatigability. Values are separated into the effect of each hypo-activity model on the upper limb (UL) *vs* the lower limb (LL). The values are taken from the references used in the text for bed-rest (LL)[64,65], immobilisation (UL)[23,66] (LL)[3,4] and ULLS (LL)[61,62]. Where there are missing bars, this shows gaps in the literature (*i.e.*, values are not available for a parameter during a specific hypo-activity model)]. Shorter periods of hypo-activity ($\leqslant$ 2 wk) generally appear to have little impact on fatigability. Muscle fatigability appears to increase in weight-bearing muscles but immobilisation in the upper body suggests an increase in resistance to fatigue. Differences between studies could be due to the duration of unloading or in the method used to test fatigue resistance. The mechanisms that cause fatigue are specific to the task being performed[68,69]. Therefore, variability between fatigue resistance responses to hypo-activity models may be due to task specificity. Studies investigating a comparison of different fatigue tasks before and after hypo-activity are sparse. Yue *et al*[12] demonstrated a task-dependent effect on muscle fatigue with substantially increased endurance time (reduced fatigability) at a low force (20% MVC) and no statistical effect at a moderate force (65% MVC) in the elbow flexors. The selective improvement of fatigue resistance for the low-force contraction was accompanied by the absence of a change in the time course of the twitch, suggesting that the immobilisation-induced adaptation included and improved efficacy of some excitation-contraction processes and underscored the major role of these mechanisms in determining the endurance time for low-force, long-duration contractions. It appears that the hypo-

activity induced adaptations in muscle fatigability vary with the specifics of the task being performed. More research is needed to investigate these task-specific responses to different models of hypo-activity.

Numerous adaptations in fatigue mechanisms have been hypothesised to explain the observed preservation and decrease in fatigability in response to hypo-activity. As stated previously, hypo-activity results in muscle atrophy and a decrease in muscle strength, have been reported to be accompanied by myofiber transitions from slow to fast[70] and a shift in fuel metabolism away from lipid fuels toward glycolysis[71]. Typically these changes are associated with increased fatigability. Cardiovascular adaptations with hypo-activity[72] reduces oxygen delivery and oxygen utilization which may impair prolonged exercise capacity. Additionally, exercise tolerance may be influenced by impaired muscle activation after hypo-activity[1,54]. In light of this, the reports of decreased fatigability with hypo-activity are puzzling, and the underlying mechanisms remain unclear. It is possible that an atrophy-induced decrease in absolute force production will result in decreased intramuscular pressure. This in turn, will increase blood flow to the muscle and increase supply to match the metabolic demand[56,73]. Other potential mechanisms include adaptations in the neural activation strategy utilised[56], adaptations in the basal inorganic phosphate concentration[74], and changes in excitation-contraction coupling[12].

NUTRITIONAL SUPPLEMENTATION

As mentioned above, there is strong evidence that protein synthesis is decreased in response to periods of bed-rest and immobilisation[40,41,43]. That resistance exercise provides an anabolic stimulus during hypo-activity is undisputed[9,59,75]. When supplemented with nutritional interventions, the benefits of exercise during bed-rest appear additive[76], thereby suggesting different synergistic pathways for counteracting atrophy. It may not always be practical to prescribe exercise to counteract the atrophy brought about by inactivity. In these cases, such as trauma, pharmaceuticals may be used and have been tried with varying degrees of success[77]. However, effective long-term medication is not a palatable option (*e.g.*, costs, side effects, repeated injections). Where exercise is not a practical prescription, supplementing the diet with potential/recognised hypertrophic nutrients may be an effective and easily adhered to intervention programme for preventing the loss of muscle mass/function seen with hypo-activity. In this latter therapeutic group, potential candidates include proteins (essential amino acids (EAAs) and Leucine in particular), creatine, omega-3 fatty acids, vitamin-D (Vit-D) and antioxidants, to name but a few[78,79].

Protein

Stuart *et al*[80] sought to determine whether the catabolic effects of bed-rest in humans was due to a decrease in protein synthesis, and if so, to assess whether increasing the amount of dietary protein might be beneficial *i.e.* The calculated non-oxidative Leucine disappearance was used as a measure of whole-body-protein synthesis, which was shown to decrease when dietary protein was low. Bed-rest resulted in a 24% decrease in nonoxidative Leucine disappearance in participants assigned to a lower-protein diet (0.6 g protein·kg body $wt^{-1}\cdot d^{-1}$), whereas Leucine kinetics were unchanged by the same bed-rest protocol in participants who received a higher-protein diet (1.0 g protein·kg body $wt^{-1}\cdot d^{-1}$)[80]. In other words, whereas protein synthesis is suggested here to decrease with bed-rest, dietary supplementation of protein appears to protect against this deleterious response.

Essential amino acids

Bolus oral ingestion of EAAs produces a several-fold increase in plasma amino acid levels[81] and has been shown to stimulate net protein synthesis to a greater extent than a mixed meal or a solution containing nonessential amino acids[82]. Studies have shown that providing a nutritional supplement enriched with EAAs could improve lean body mass, strength and physical function even without exercise[83]. Previous studies by Stein *et al*[84,85] have shown improved nitrogen balance during both 6 and 14 d of bed-rest when provided with a daily supplementation of 11 g of branch-chain amino acids (BCAA), compared with the same dose of nonessential amino acids. It appears that a greater dose of EAAs (49.5 g/d) during 28 d bed-rest prevented any noticeable changes in muscle mass[86]. Paddon-Jones *et al*[86] however, reported that during this 28 d period that although no changes in muscle mass were observed they did find a decline in muscle strength. Nonetheless, the decrease in muscle strength with EAAs (11%) was still noticeably less than the decrease in strength seen in the control group (23%)[86]. These results collectively demonstrate a positive effect of EAAs supplementation during periods of bed-rest ranging from 6 to 28 d on both muscle mass and function[84-86].

Creatine

Creatine supplementation is another potential supplement that may attenuate hypo-activity induced decreases in muscle size and strength. Johnston *et al*[87] reported that short-term (29 d) creatine supplementation (20 g/d) attenuates the loss in muscle mass and strength during upper arm immobilisation. It is well known that muscle total creatine content can be rapidly raised by a high-dose oral creatine intake[88] and that long-term creatine intake can enhance the effects of weight training on muscle size and strength[89,90]. Creatine supplementation during 10 wk of resistance training has been shown to accelerate the rate of muscle hypertrophy in young adults who previously had their knee flexors immobilised for 2 wk[91]. Furthermore, 14 d creatine supplementation during hind-limb immobilisation lessened the rate of loss in the plantarflexors in a rodent model[92]. Additionally, Op't Eijinde *et al*[93] showed that creatine supplemen-

tation prevented the loss of glucose transporter type 4 (GLUT4) during muscle disuse and increased muscle GLUT4 content above normal levels during subsequent rehabilitation. Collectively these studies suggest that creatine supplementation during resistance training and rest may be effective at reversing or maintaining lower-body muscle mass during and after an immobilised state.

Antioxidants

Intricate antioxidant defence systems in the body work to continually manage oxidative stress. To counteract ROS, enzymatic and nonenzymatic antioxidants work together[94]. Enzymes work to improve or maintain an antioxidant balance and to avert oxidative damage by scavenging or preventing transformation of ROS to intracellular molecules and inhibiting their conversion to more deleterious forms. Endogenous nonenzymatic antioxidants such as vitamins-C and -E, carotenoids and flavonoids play important roles by contributing to the antioxidant system as cofactors for antioxidant enzymes. Results from Zwart *et al*[95] provide evidence that increased oxidative stress occurs during bed-rest. These data are also supported by results of several other studies that show evidence for elevated oxidative stress and increased ROS[96-98]. It would be interesting to see whether antioxidant supplementation during hypo-activity models will have beneficial effects on these outcome measures and furthermore, see whether this would then result in the attenuation of muscle loss in these models.

Vitamin-D

Ceglia proposed Vit-D supplementation as an effective nutritional intervention to attenuate age related sarcopenia[99]. Vit-D supplementation (800 IU per day) for periods of 8 to 12 wk has been reported to reduce postural sway and improve the risk of falling in elderly individuals[100,101]. Longer periods (12 mo) of Vit-D supplementation (800 IU per day) in the elderly has been shown to increase strength, decrease body sway and increase physical performance[102]. However, in a healthy elderly population with no Vit-D deficiency Vit-D supplementation does not appear to improve muscle strength or function[103,104]. It remains to be seen whether Vit-D supplementation in healthy persons with no Vit-D deficiency, any enhancement in muscle structural or contractile properties can be attained in the presence of hypo-activity.

Omega-3 (EPA)

Recent studies by Smith *et al*[105,106] supplemented healthy young and elderly individuals with omega-3 fatty fish-oils for 8 wk and found a significant increase in the muscle protein synthetic response to amino acid administration. They concluded in the elderly model that omega-3 fatty acids might be useful for the prevention and treatment of sarcopenia[105]. Dietary fish oil has also been shown to alleviate soleus muscle atrophy during immobilisation in association with Akt signalling in rats[107]. It would therefore seem reasonable to suggest that more investigation is needed into the potential of omega-3 fatty acids as a nutritional supplement for attenuating muscle atrophy with hypo-activity. In parallel, it is believed that omega-3 fatty acids may impact on lean body mass though decreasing the effectiveness of catabolic cytokines, reduced protein degradation and improving insulin sensitivity[108]. There is evidence to suggest that eicosapentaenoic acid (EPA) an omega-3 fatty acid may reduce the pro-inflammatory cytokines associated with inflammation[109]. Magee *et al*[109] demonstrated *in vitro* that EPA inhibits the effects of TNF-α by reducing its apoptotic effects and enabling myogenesis. It is however debatable whether this supplement would be useful in combating muscle atrophy where, as seen in human hypo-activity models, there is scant evidence for increased protein breakdown[40].

CONCLUSION

Hypo-activity models result in profound changes in skeletal muscle morphology and strength. Muscle mass and strength losses vary between different hypo-activity models, with immobilisation causing the most profound decreases, greater than bed-rest and limb suspension. Decrements in muscle size and strength are seen in response to hypo-activity models with the greatest decrements seen in antigravity muscles. The decreases in strength seen with hypo-activity models surpass the losses in muscle mass and as such, the nervous system and contractile properties adapt to adjust for this excessive loss of strength. Nutritional supplementation may stand as a viable intervention to combat muscle atrophy with hypo-activity when exercise is not a practical prescription. There are several potential nutritional supplements that could be used to combat muscle atrophy but extensive research is needed to determine the most affective.

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OMEGA-3 FATTY ACIDS AND VITAMIN D IN IMMOBILISATION: PART A- MODULATION OF APPENDICULAR MASS CONTENT, COMPOSITION AND STRUCTURE

E.L. BOSTOCK, C.I. MORSE, K. WINWOOD, I.M. MCEWAN, G.L. ONAMBÉLÉ

Health, Exercise and Active Living Research Centre, Department of Exercise & Sport Science, Manchester Metropolitan University, Crewe Green Road, Crewe, CW1 5DU, United Kingdom. Corresponding author: Gladys Onambele-Pearson, Health, Exercise and Active Living Research Centre, Department of Exercise & Sport Science, Manchester Metropolitan University, Crewe Green Road, Crewe, CW1 5DU, United Kingdom. Tel: +44 (0) 161 247 5594; Email: g.pearson@mmu.ac.uk

Abstract: *Objectives:* Muscle size decreases in response to short-term limb immobilisation. This study set out to determine whether two potential protein-sparing modulators (eicosapentaenoic acid and vitamin D) would attenuate immobilisation-induced changes in muscle characteristics. *Design:* The study used a randomised, double-blind, placebo-controlled design. *Setting:* The study took part in a laboratory setting. *Participants:* Twenty-four male and female healthy participants, aged 23.0±5.8 years. *Intervention:* The non-dominant arm was immobilised in a sling for a period of nine waking hours a day over two continuous weeks. Participants were randomly assigned to one of three groups: placebo (n=8, Lecithin, 2400 mg daily), omega-3 (ω-3) fatty acids (n=8, eicosapentaenoic acid (EPA); 1770 mg, and docosahexaenoic acid (DHA); 390 mg, daily) or vitamin D (n=8, 1,000 IU daily). *Measurements:* Muscle and sub-cutaneous adipose thickness (B-mode ultrasonography), body composition (DXA) and arm girth (anthropometry) were measured before immobilisation, immediately on removal of the sling and two weeks after re-mobilisation. *Results:* Muscle thickness (-5.4±4.3%), upper and lower arm girth (-1.3±0.4 and -0.8±0.8%, respectively), lean mass (-3.6±3.7%) and bone mineral content (BMC) (-2.3±1.5%) decreased significantly with limb immobilisation in the placebo group ($P<0.05$). Despite no significant effect of group, ω-3 and vitamin D supplementation showed trends ($p>0.05$) towards attenuating the decreases in muscle thickness, upper/lower arm girths and BMC observed in the placebo group. The ω-3 supplementation group demonstrated a non-significant attenuation of the decrease in DXA quantified lean mass observed in the placebo group. Sub-cutaneous adipose thickness increased in the placebo group ($P<0.05$). ω-3 and vitamin D both blunted this response, with ω-3 having a greater effect ($P<0.05$). All parameters had returned to baseline values at the re-mobilisation phase of the study. *Conclusion:* Overall, at the current doses, ω-3 and vitamin D supplementation only attenuated one of the changes associated with non-injurious limb immobilisation. These findings would necessitate further research into either a) supplementation linked to injury-induced immobilisation, or b) larger doses of these supplements to confirm/refute the physiological reserve potential of the two supplements.

Key words: Docosahexaenoic acid, eicosapentaenoic acid, immobilisation, lecithin, omega 3, vitamin D.

Introduction

Skeletal muscle undergoes structural adaptations in response to changes in functional demand. Prolonged periods of reduced muscle activity and mechanical loading, e.g. immobilisation, limb-suspension or bed-rest, result in changes in skeletal muscle structure and function, bone mineral density (BMD) and intermuscular adipose content (1-6). Musculoskeletal trauma and sports injuries are often treated with orthopaedic surgery/ limb immobilisation, which inevitably leads to periods of inactivity and disuse. The prognosis of orthopaedic surgery patients is poor especially in the older population with an ~6 to 17% mortality rate within three years of hip and knee joint replacement (7). Exercise could be beneficial in these circumstances, but is not always practical. In addition, there is a notable need to identify non-pharmacological interventions since polypharmacy in itself is conducive to skeletal tissue loss (8).

Eicosapentaenoic acid (EPA) is an n-3 polyunsaturated fatty acid with anti-inflammatory properties, which is synthesised from ingested alpha-linolenic acid or consumed in fish, or in fish oil, such as sardines and cod liver oil. Despite there being no established Dietary Reference Intake for n-3 fatty acids, adequate intake (AI) is set at 1.6 and 1.1 g/day for men and women, respectively (9). There is evidence to suggest that EPA may reduce the pro-inflammatory cytokines associated with muscle damage-induced inflammation (10). Magee et al. (2008) demonstrated in vitro that EPA inhibits the effects of TNF-α by reducing its apoptotic effects and enabling myogenesis (10). It is unclear whether this supplement would have a beneficial effect during immobilisation, where it is generally accepted that there is muscle atrophy (11), which is associated with decreased protein synthesis [12] but scant evidence for increased protein breakdown (13).

Another non-pharmacological agent that may potentially be used against the asthenia and sarcopenia induced through immobilisation is vitamin D. Vitamin D is required to absorb calcium and phosphorus and plays a crucial role in maintaining bone, muscle function, modulation of cell growth, neuromuscular and immune functions, and reduction of

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inflammation. The main source of vitamin D is sunlight, with smaller amounts found in certain foods. The Recommended Dietary Allowance for vitamin D is 600 IU/day (14). Vitamin D supplementation is another way of making sure the recommended allowance is achieved. Vitamin D has been shown to have direct effects on muscle (15); however, the exact mechanisms remain unknown. To date, research has indicated an association between genetic variation in the vitamin D receptor gene and muscle strength, fat mass and body mass in premenopausal women (16). Moreover, vitamin D has been reported to impact on both the trans-membranous flows of calcium and phosphate in skeletal muscle, and the synthesis rate of contractile properties (17). Vitamin D supplementation reduced falls by 49% and improved musculoskeletal function in frail elderly women with vitamin D deficiency (18). It remains to be seen whether vitamin D supplementation in healthy persons with no known vitamin D deficiency, would lead to any enhancement in muscle structural and contractile properties in the presence of immobilisation.

In the present study, an arm immobilisation model was chosen as it is relatively less restrictive on daily life and causes fewer burdens on participants. The aim was to determine the role that the two potential protein-sparing modulators (EPA or vitamin D supplementation) may play in attenuating atrophy induced through a model that would emulate relatively short-term decreased local mobility/activity in humans. The differential effect of immobilisation on in vivo muscle (thickness), limb composition (lean mass, bone parameters and fat mass), and anthropometry (arm girth) was systematically monitored. Participants received either omega 3 (ω-3, a fish oil of a complex of EPA and docosahexaenoic acid (DHA)), vitamin D or placebo (Lecithin), hereafter simply referred to as EPA, vitamin D or placebo supplementation. It was hypothesised that muscle thickness, lean mass and arm girth will decrease with limb immobilisation. It was also hypothesised that EPA will be the most effective supplement at minimising these changes, since it is understood to act on the protein synthesis pathways.

Method

Participants

Twenty-four healthy volunteers were recruited from the local university campus. All participants provided written informed consent before taking part in this study, which was approved by the local Ethics Committee of Manchester Metropolitan University. Exclusion criteria were any conditions requiring the use of medication likely to affect muscle function or musculoskeletal health (e.g. statins and oral steroids), and any current or history of kidney/liver disease, as those suffering with such conditions are more susceptible to the side effects of nutritional supplementation. An in-house designed physical activity and general health questionnaire, generally used in our research laboratories for teaching and research purposes, was completed by each participant, to ascertain health, habitual physical exercise levels and supplement history prior to the study. This questionnaire confirmed all participants were recreationally active (defined here as undertaking three hours or less of low-to-moderate intensity exercise per week.), free from recent (last 6 months) upper limb injury, were not currently taking any supplements and had no history of ω-3 or vitamin D supplementation within at least the last year. Participants were randomly assigned to one of three groups: placebo [PLA: n = 8]; EPA [EPA: n = 8]; or vitamin D [Vit-D: n = 8]. For anthropometric measurements (height, weight and BMI) in our three populations, please see the results section.

Study design

The study used a randomised, double-blind, placebo-controlled design. All participants attended a familiarisation session at least one week prior to the first testing session. All testing sessions were completed after an overnight fast. After baseline testing, the non-dominant arm was immobilised in a sling, with the correct sling wearing procedure demonstrated to each participant (Figure 1). Participants were required to wear the sling for nine waking hours a day for two continuous weeks, removal of the sling was permitted only when necessary (e.g. taking a bath/shower, driving, sleeping etc.), minimising any movement medio-laterally at the elbow and shoulder, whilst requiring participants to not contract the upper musculature (including the hands) during the hours of immobilisation. Measures of upper arm muscle and subcutaneous fat thickness, body composition (lean mass, bone parameters and fat mass), and upper and lower arm girth were taken immediately before immobilisation (Pre), on removal of the sling (Post), and two weeks after re-mobilisation (Post2).

The placebo group consumed two 1200 mg capsules of Soya Lecithin (Holland & Barrett, UK) a day, each daily dose typically providing 1464 mg of Phosphatides. The EPA group consumed three softgel of High EPA Formula (MorEPA, Minami Nutrition, UK), with the daily dose providing 1770 mg EPA and 390 mg docosahexenoic acid (DHA). The Vit-D group consumed one softgel of Vitamin D3 (Now Foods, Bloomingdale, U.S.A.), each dose providing 1,000 IU of Vitamin D3. The participants consumed the nutritional supplements during the two weeks of limb immobilisation. Participants were asked to maintain their habitual diet and not to perform any unaccustomed strenuous exercise during the 2 weeks of immobilisation and remobilisation. To monitor this, during the immobilisation period participants completed a 3-day food diary, a daily activity log (including sling-wear hours) and wore a pedometer (Omron Walking style III step counter, Omron Healthcare Co., Ltd, Kyoto, Japan) to record the number of steps taken each day. The food diaries were analysed for macronutrient and micronutrient average intake using Microdiet Plus 1.2 (Microdiet, Downlee Systems Ltd, UK). All participants recorded their daily sling wear hours. In addition, since they were university-based local recruits, they

were in full view of at least one member of the research team and/or other university staff on campus who had been made aware of the study requirement, during their hours on campus. Last but not least, participants were questioned during their final testing session, to confirm their compliance with the protocol.

Muscle and sub-cutaneous adipose thickness measures

All images were recorded after approximately 20 minutes seated rest to avoid fluid shifts that might induce interstitial and/or intracellular changes (19). Images of the muscle and sub-cutaneous adipose tissue of the upper arm were obtained using B-mode ultrasonography (AU5, Esaote, Genoa, Italy). A 7.5-MHz linear phased-array probe (image depth: 37.1-92.8mm) was applied in the sagittal plane with minimal pressure to the tissue area of interest to avoid image distortion. This method has previously been shown to be highly reliable for determining muscle and adipose thickness (20-22). Images were recorded using Adobe Premiere 6.0 (Adobe Systems, USA) and stored for later analysis.

Images were obtained with the participant in an upright, seated position with their arm abducted square to the body (elbow at 180°) and resting on the ultrasound machine. In the upper arm, the proximal and distal insertions of the biceps and triceps brachii were identified by sonography and marked on the skin. The midpoint (L50) and a third of the distance (L33) from the distal end of the biceps and triceps brachii were identified and marked onto the skin. Upper arm ultrasonography images were collected in the sagittal plane, at both sites on both the biceps and triceps brachii. Muscle thickness was measured as the distance from the top of the superficial muscle aponeurosis to the bone at both sites along the biceps and triceps brachii. Ultrasound assessment of muscle tissue content has previously been validated (23-26). Sub-cutaneous adipose thickness was measured as the distance from the bottom of the epidermis to the top of the superficial muscle aponeurosis in the biceps and triceps at both sites. Each of these distances were measured at three standardised points along the width of the probe to obtain average muscle and sub-cutaneous adipose thicknesses using ImageJ analysis software (ImageJ 1.37, Maryland, USA).

Body composition analysis

Body composition was determined using dual-energy X-ray absorptiometry (DXA) scanner (Hologic Discovery; Vertec Scientific, Reading, Berkshire, UK). Whole body scans were performed lasting approximately seven minutes with a dose-area product (DAP) of 21 cGy*cm^2. The appendicular mass was isolated from the trunk and head using DXA regional computer-generated default lines, with manual adjustment, on the anterior view planogram. Measures of BMD, bone mineral content (BMC), lean mass, fat mass and fat percentage are reported for the immobilised arm only.

Arm girths

Participants assumed a relaxed standing position with arms hanging by the sides and palms facing the hips. A measuring tape was used to measure upper arm girth at the mid-acromial-radial and lower arm girth at a fixed point a third of the way (from the proximal end) along the length of the radiale-stylion. Measurements were repeated three times at each point and average girths were calculated.

Measurement reliability

All protocols were assessed for intra as well as inter-day reliability. This utilised five participants and entailed carrying out measurements three times on day 1, and repeating these on day 2, approximately a week later. Within-day coefficient of variation (CV) of 0.1 %, 0.1 %, 0.4 %, 0.2 %, 0.3 % and 0.3 %, and between-day CVs of 0.4 %, 0.2 %, 0.6 %, 0.2 %, 0.2 % and 0.4 % were yielded for upper arm girth, lower arm girth, biceps muscle thickness, triceps muscle thickness, biceps subcutaneous adipose thickness and triceps subcutaneous adipose thickness, respectively. DXA reliability is reported with a CV of 1.0 %.

Statistical analyses

Data were analysed using IBM SPSS v21 (IBM Inc, USA). The Shapiro-Wilk test revealed some of the data to be non-parametric (upper arm muscle and adipose thickness, upper and lower arm girths). The effect of immobilisation was examined by assessing the changes seen in the PLA group by either repeated measures ANOVA (parametric data) or a Friedman test (non-parametric data). Parametric change relative to baseline values (Pre-to-Post: (Post-Pre)/Pre; and Pre-to-Post2: (Post2-Pre)/Pre) were analysed using a repeated measures ANOVA, with post-hoc Bonferonni corrected 2-tailed t-tests to determine group difference. Non-parametric between group effects on change data were analysed using the Kruskal Wallis test, with post-hoc Mann-Whitney U tests. All data are presented as mean ± standard deviation (SD). Statistical significance was set with alpha at ≤ 0.05.

Results

The non-immobilised limb was also monitored throughout the immobilisation and supplementation phase. When the immobilised limb data was normalised for the non-immobilised limb data there was no effect on the outcome measures. Data for the non-immobilised limb is therefore not reported in this paper.

Homogeneity of sample

There were no significant differences in baseline characteristics between the groups (Table. 1). The groups similarly did not differ in baseline muscle size (e.g. bicep muscle thickness (L50) - PLA: 28.7 ± 7.1 mm; EPA: 31.1 ± 5.4 mm; Vit-D: 32.1 ± 6.5 mm), arm girth (PLA: 28.8 ± 3.0 cm;

EPA: 28.1 ± 4.0 cm; Vit-D: 29.4 ± 3.2 cm), body composition (e.g. lean mass – PLA: 1874.5 ± 595.8 g; EPA: 2361.0 ± 1116.8 g; Vit-D: 2531.1 ± 832.6 g).

Table 1
Baseline characteristics of all participants

	PLA	EPA	Vit-D
Age (years)	26 ± 6.7	19 ± 1.6	23 ± 5.9
Gender			
Males	n = 2	n = 4	n = 3
Females	n = 6	n = 4	n = 5
Height (cm)	168.7 ± 11.1	169.5 ± 12.0	172.2 ± 8.0
Weight (kg)	69.1 ± 14.2	69.6 ± 23.1	75.2 ± 14.5
BMI	24.1 ± 3.7	24.1 ± 5.1	24.4 ± 5.0

Mean values ± SD for baseline characteristics.

Figure 1
Image demonstrating correct sling wear

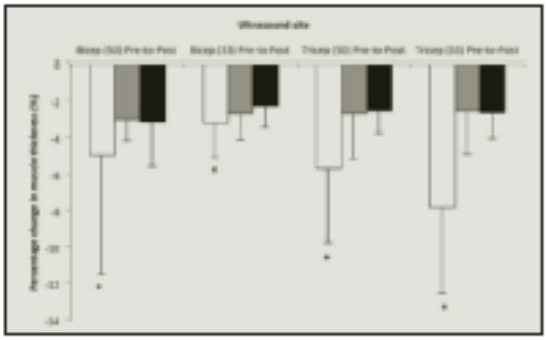
Front, side and back view demonstrating sling wear. Sling wear was initially demonstrated by the experimenter and on subsequent applications, sling application was self-administered by each participant. The sling was worn for a minimum of nine waking hours a day for 14 days. The elbow joint was positioned at approximately a 90° angle.

Daily physical activity and nutritional intake

No significant differences were observed in calorific intake (PLA: males 3134 ± 247 kcal/day and females 1352 ± 506 kcal/day; EPA: males 1826 ± 780 kcal/day and females 1468 ± 121 kcal/day; Vit-D: males 2658 ± 679 kcal/day and females 1541 ± 431 kcal/day ($p>0.05$)) or in habitual physical activity (PLA: males 8216 ± 34 steps/day and females 6361 ± 1681 steps/day; EPA: males 9648 ± 2934 steps/day and females 5410 ± 2231 steps/day; Vit-D: males 7795 ± 1050 steps/day and females 5156 ± 1940 steps/day ($p>0.05$)) between the groups during the course of the immobilisation. This effect was true for the EPA, Vit-D and PLA groups. Further diet composition analyses revealed no group differences in protein (PLA: 1.1 ± 0.3 g·kg-1·bw/day; EPA: 1.0 ± 0.3 g·kg-1·bw/day; Vit-D: 1.0 ± 0.6 g·kg-1·bw/day), carbohydrate (PLA: 3.1 ± 1.5 g·kg-1·bw/day; EPA: 3.1 ± 1.1 g·kg-1·bw/day; Vit-D: 3.0 ± 1.0 g·kg-1·bw/day), fat (PLA: 1.0 ± 0.5 g·kg-1·bw/day; EPA: 1.0 ± 0.5 g·kg-1·bw/day; Vit-D: 1.0 ± 0.3 g·kg-1·bw/day), vitamin D (PLA: 1.7 ± 0.7 ug/day; EPA: 1.6 ± 0.9 ug/day; Vit-D: 2.0 ± 1.6 ug/day) or ω-3 (PLA: 0.37 ± 0.30 g/day; EPA: 0.36 ± 0.19 g/day; Vit-D: 0.36 ± 0.20 g/day) intake over the immobilisation period between the three groups.

Results of the questionnaire revealed that participants walked to and from work, university and/or shopping a minimum of 30 minutes per day. Examination of the activity diaries completed by the participants themselves during the immobilisation period, confirmed that the majority of participants spent at least half an hour outside each day.

Figure 2
Muscle thickness changes in response to immobilisation and supplementation



Percentage change (Mean difference (%) ± SD) in muscle thickness from Pre-to-Post for PLA (white bars), EPA (grey bars) and Vit-D (black bars) at the midpoint (L50) and a third of the distance (L33) along the length of the biceps and triceps brachii. * Significant difference between Pre and Post immobilisation in the PLA group.

Muscle and sub-cutaneous adipose thickness measures

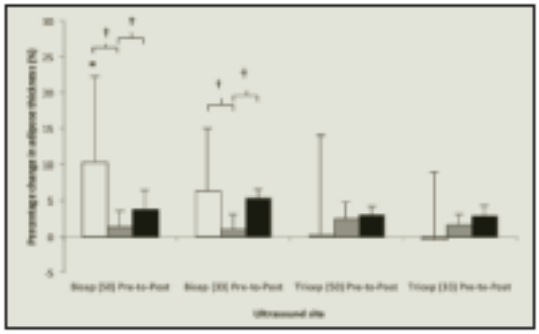
Muscle thickness decreased from Pre to Post at both sites (L50 and L33) on the biceps and triceps brachii ($p<0.05$) in the placebo group. The percentage decrease in muscle thickness was significantly greater in the triceps brachii (L50 & L33) than the biceps brachii (L33) ($p<0.05$) The percentage change in muscle thickness at both sites on the biceps and triceps brachii did not significantly differ between the groups (Figure. 2), though both EPA and Vit-D groups showed a non-significant trend towards differing from the PLA group. Sub-cutaneous adipose thickness increased significantly post immobilisation at L50 of the biceps brachii ($p<0.05$), with no significant change at L33 of the biceps brachii or either site on the triceps brachii. The percentage change in adipose thickness was not significantly different between groups at both sites on the triceps brachii (Figure 3). Percentage change in adipose thickness was significantly greater in the PLA than the EPA group ($p<0.05$) and in the Vit-D than the EPA group ($p<0.05$) at L50 and L33 along the biceps brachii.

Body composition

There was a significant decrease in lean mass Post immobilisation ($p<0.05$) but no significant effect of supplement group on the percentage change in lean mass (Figure 4). Percentage changes in BMC, BMD, fat mass and fat percentage

are displayed in Table 2. No significant change was observed in BMD, fat mass or fat percentage. There was a significant decrease in BMC from Pre to Post immobilisation in the PLA group ($p<0.05$). There was no significant difference in percentage change in BMC, BMD, fat mass or fat percentage between the groups Post or Post2 ($p>0.05$).

Figure 3
Sub-cutaneous adipose thickness changes in response to immobilisation and supplementation

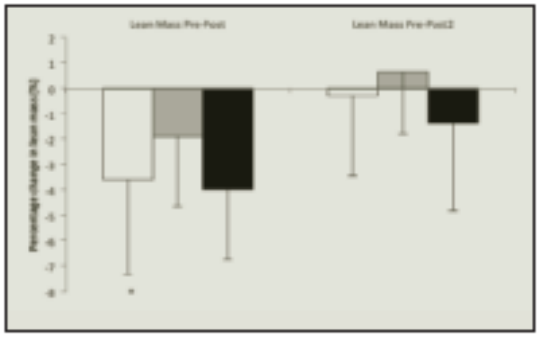


Percentage change (Mean difference (%) ± SD) in adipose thickness from Pre-to-Post for PLA (white bars), EPA (grey bars) and Vit-D (black bars) at the midpoint (L50) and a third of the distance (L33) along the length of the biceps and triceps brachii. * Significant difference between Pre and Post immobilisation in the PLA group. † Significant difference in % change between groups.

Arm girths

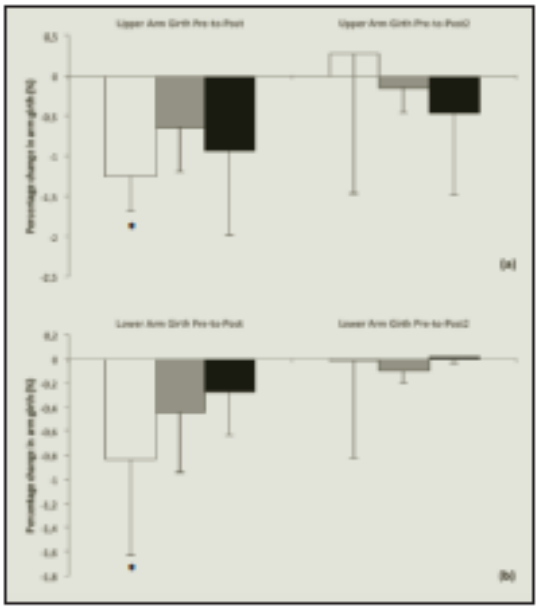
Upper and lower arm girths significantly decreased from Pre to Post immobilisation ($p<0.05$) in the PLA group. There was no significant difference in the percentage change in upper or lower arm girth between groups (Figure 5).

Figure 4
Lean mass changes in response to immobilisation and supplementation



Percentage change (% ± SD) in lean mass from Pre-to-Post and Pre-to-Post2 for PLA (white bars), EPA (grey bars) and Vit-D (black bars). * Significant difference between Pre and Post immobilisation in the PLA group.

Figure 5
Arm girth changes in response to immobilisation and supplementation



Percentage change (% ± SD) in upper (a) and lower (b) arm girth from Pre-to-Post and Pre-to-Post2 for PLA (white bars), EPA (grey bars) and Vit-D (black bars). * Significant difference between Pre and Post immobilisation in the PLA group.

Discussion

The purpose of this study was to determine the role that two potential protein-sparing modulators (EPA or vitamin D supplementation) may play in attenuating the deleterious physiological changes induced through 2 weeks of 9-waking-hours-per-day combined arm and shoulder immobilisation. We hypothesised that muscle thickness, lean mass and arm girth would decrease with limb immobilisation. We found evidence to support this, with significant decreases in muscle thickness (PLA: -3.2 to -7.8% dependent on anatomical site), arm girth (PLA: -0.8 to -1.3% dependent on anatomical site) and lean mass (PLA: -3.6% for the immobilised limb). In addition, we found a significant increase in sub-cutaneous adipose thickness (PLA: 10.3%) and a significant decrease in BMC (PLA: -2.3%). It was also hypothesised that EPA would be the most effective supplement at minimising the effects of immobilisation. In fact, our data show that neither EPA nor vitamin D had any significant effect on the responses to non-injurious immobilisation, other than on the associated accumulation of sub-cutaneous fatty tissue. Nonetheless, a few trends towards attenuations in deleterious physiological events were observed, in the EPA and Vit-D treated groups.

Table 2
Changes in immobilised limb composition in response to immobilisation and supplementation

	PLA		EPA		Vit-D	
	Pre-to-Post	Pre-to-Post2	Pre-to-Post	Pre-to-Post2	Pre-to-Post	Pre-to-Post2
BMC	-2.3 ± 1.5 *	-1.5 ± 1.1	-0.3 ± 1.0	0.6 ± 1.9	-0.7 ± 1.9	-0.6 ± 0.7
BMD	-1.6 ± 2.6	0.4 ± 2.2	-0.5 ± 3.2	-0.3 ± 1.1	0.0 ± 1.5	0.5 ± 2.1
Fat mass	2.0 ± 2.4	-1.4 ± 6.7	3.2 ± 3.2	0.2 ± 1.7	2.0 ± 3.5	1.9 ± 1.8
Fat %	2.1 ± 5.9	0.0 ± 5.2	2.6 ± 5.3	-1.2 ± 4.3	3.4 ± 2.6	0.4 ± 3.6

Percentage change (% ± SD) in immobilised limb composition parameters from Pre-to-Post and Pre-to-Post2 for PLA, EPA and Vit-D. * Significant difference between Pre and Post immobilisation in the PLA group.

We discuss the observed trends for the attenuation of some parameters.

A significant change in upper limb muscle thickness is evidenced in our ultrasound data, with a significant decrease in biceps and triceps brachii muscle thickness with immobilisation. The ultrasound data demonstrate a greater decrease in triceps brachii muscle (L50 and L33) thickness than the biceps brachii (L33) in the PLA group. We had, in fact, expected the muscle held in the shortened position (i.e. the biceps) to be impacted on more than the muscle held in the lengthened position (i.e. the triceps); previous research suggests that sarcomeres are added in series when the muscle is immobilised in the lengthened position, and sarcomeres are lost when the muscle is immobilised in the shortened position (27). In the present study, the elbow was immobilised at a 90° angle and, as such, would not have exerted maximal lengthening or shortening on the triceps and biceps brachii. The EPA and Vit-D supplementation groups demonstrated non-significant trends towards smaller decreases in muscle thickness in response to immobilisation, suggesting that EPA and vitamin D may have some role to play in attenuating muscle atrophy associated with disuse. A recent study in an animal model demonstrated distinct effects of EPA and DHA on protein metabolism (protein synthesis and breakdown) with EPA showing a greater ability to result in skeletal muscle protein accretion. Further work in humans is required to further investigate this and the effects EPA may have in human disuse models. Indeed, it is likely that the dose, duration and/or whole body measurement of muscle mass that was adopted in these animal studies diminished the comparability of animal models from our immobilisation model.

Upper and lower arm girths were shown to decrease significantly post immobilisation in the PLA group. We used girth as a gross marker of skeletal muscle atrophy, as previously used by Matsumura et al (28). A decrease in upper arm girth (in the PLA group) suggests a decrease in muscle CSA in the upper arm and this supports our ultrasound data along with previous findings of a decrease in elbow flexor muscle CSA and volume with arm immobilisation (29). The decrease in lower arm girth suggests a decrease in forearm muscle CSA, and this is in line with finding from Miles et al. who reported a decrease in forearm muscle CSA with 9 days arm casting (30). The significant decrease in limb CSA is reflected in our DXA data, with a significant decrease in lean mass with immobilisation. The decrease in upper arm girth appeared to be attenuated by EPA and vitamin D supplementation, with EPA having the greater effect. Similarly, in the lower arm, supplementation showed a trend towards attenuating the losses in arm girth, this time with vitamin D having a slightly greater effect. The EPA group appear to show a smaller decrease in lean mass than both other groups.

The ultrasound data revealed no significant change in sub-cutaneous adipose thickness in the triceps brachii over the immobilisation period. Biceps sub-cutaneous adipose thickness, however, did significantly increase at the midpoint site in the placebo group. Manini et al. (6) reported a significant increase in intermuscular adipose tissue and no significant change in subcutaneous adipose tissue in response to 4-weeks unilateral lower limb suspension. The difference between the response of sub-cutaneous adipose tissue to disuse in the current study in comparison to Manini et al. may be due to the different techniques used to assess the parameter (i.e. ultrasound vs. magnetic resonance imaging) and/or the differing modes of immobilisation (i.e. arm immobilisation vs. unilateral lower limb suspension). It is possible that the more stringent immobilisation in their study (throughout daily mobile activities, for four weeks), as well as the adiposity site (intermuscular), may account for the fact that they observed significant increases in adiposity. The present study partially agrees with their findings as our data showed a significant increase at one region, subcutaneously, along the upper arm (i.e. 50% of upper limb length). The EPA group demonstrated a significantly smaller increase in biceps sub-cutaneous adipose thickness following immobilisation than both the PLA and Vit-D groups. This effect may, indeed, be meaningful given that diet was monitored and the dietary records demonstrated neither time nor between group differences in total calories, macronutrients or vitamin D intakes, throughout the study. EPA supplementation potential for attenuating increases in sub-cutaneous adipose thickness in response to disuse. The Vit-D group also demonstrated a trend towards an attenuation in the increase of sub-cutaneous adipose thickness in comparison to

the PLA group.

DXA analysis revealed no significant changes in fat mass or fat percentage with limb immobilisation. The difference in the response of adiposity to disuse in the current study in comparison to Manini et al. (6), who reported a significant increase in intermuscular adipose tissue, may be due to the different techniques used to assess the parameter (i.e. DXA vs. magnetic resonance imaging) or the different mode and/or duration of disuse (i.e. 2 weeks arm immobilisation vs. 4 weeks unilateral lower limb suspension). Interestingly, the increases we reported in sub-cutaneous adipose thickness were not matched by our DXA analysis of fat mass or percentage. Possible explanations for this include: a) the DXA takes the average of the whole limb rather than regional data and hence would have averaged-out the regional changes observed with the ultrasound; b) it is possible that the tissue density changes with immobilisation mean that the DXA may have miss-qualified muscle and fat after immobilisation (31).

We reported no significant change in BMD (-0.8%/week), but a significant decrease in BMC (-1.2%/week) following immobilisation. Decreases in bone are often reported in more severe and longer periods of disuse. For example, Rittweger et al. reported significant decreases in BMC of the tibia (-0.1 to -0.5/week) and radius (-0.03 to -0.05%/week) in response to 90 days bed rest (5). Marchetti et al. found significant decreases in BMD (-1.0 to 2.3%/week) in response to 6 weeks arm immobilisation, however, the participants had also undergone surgery, which could contribute to greater decreases in BMD (32). Values for BMC and BMD changes with immobilisation showed a trend towards attenuation of changes with EPA and vitamin D supplementation. Previous research indicates that BMD is affected by changes in body weight and composition (33), therefore our observed changes in muscle and sub-cutaneous adipose thickness may have influenced our BMD values. The value of BMD can also be limited by the inherent limitations of using a two-dimensional x-ray projection to estimate bone area and geometrical changes. The use of peripheral quantitative tomography (pQCT) may have been more advantageous to examine these specific changes.

A recent study in our own laboratory, found that a lower dose of EPA and DHA than that used in the current study, was not sufficient to decrease inflammation (34). We therefore chose to increase the EPA and DHA of our ω-3 supplement in the current study, in line with previous levels used in the literature (35-37). The vitamin D dose of 1000 IU is equal to 25 ug. Public Health England suggest that adults at risk of vitamin D deficiency should take a daily supplement containing 10 ug of vitamin D (38). Our selected dose is much higher than this and as such should be sufficient in the healthy population used in the current study. The formula of each supplement was chosen so that the dose provided would be able to be bought over the counter and be taken without prescription. The lack of the effectiveness of vitamin D and EPA in the current study may be due to the dosage of the supplements used and therefore this needs to be altered, potentially increased, in future studies.

The NHS consensus on vitamin D states that if people achieve a sufficient supply of vitamin D in the summer, most should keep levels greater than the deficiency threshold of 25nmol/l in winter even without supplements (39). In the current study, participants reported that they typical spent at least 30 minutes outdoors every day of the week and as such would reach the necessary sunlight exposure for adequate vitamin D levels. Research has shown that light of even relatively low intensity (~180 lux) significantly phase-shifts the human circadian rhythm [40]. We therefore suggest that the current population are unlikely to be vitamin D deficient. The "healthy, presumably non-deficient" status of the participants may have been another reason for the lack of effectiveness of the supplementation in the current study. In a recent study, it was reported that in an elderly population, most participants were unable to meet the recommended daily omega-3 (ω-3) intake without the use of fish oil supplements (41). Future studies need to recruit those lacking in physiological reserve and those more at risk of malnutrition/deficiencies e.g. older and/or injured persons. Future studies also need to assess endocrine markers, including serum EPA & DHA, 25(OH) D3 levels (biomarker for vitamin D status) and parathyroid hormone (PTH) to observe the effects of supplementation.

Blinding of ω-3 supplementation is sometimes an issue in studies due to the fact that it often repeats on participants. On completion of the study, participants were asked whether they were able to identify what supplement they believed they were taking. None of the participants correctly guessed the supplement they were taking and in fact, two participants receiving the placebo supplement believed they were taking an ω-3 supplement.

Conclusion

In accordance with previous research, we demonstrated a decrease in muscle thickness, arm girth and lean mass with short-term upper limb immobilisation. We also observed a significant decrease in BMC, with no observed effect of immobilisation on fat mass or BMD. At the current dosage, neither vitamin D nor EPA supplementation impacted on these parameters at a statistically significant level. Interestingly nonetheless, we have observed that in the case of sub-cutaneous adiposity on the biceps brachii, there is a protective effect of EPA supplementation against limb immobilisation. We propose that the model used in the current study could have relevance to a sporting population in which short-term immobilisation may be prescribed (e.g. treatment for minor injury) or in clinical populations (e.g. injury/surgery induced short-term immobilisation/bed-rest). A short-term model such as this is relatively less invasive and inconvenient for participants and therefore provides a model to be used to assess other supplements and treatments in future studies.

Ethics declaration: All experimental procedures were conducted in accordance with

the guidelines in the Declaration of Helsinki and approved by the Ethics Committee of Manchester Metropolitan University.

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Conflict of Interest Disclosure: The authors do not have any conflicts of interest.

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OMEGA-3 FATTY ACIDS AND VITAMIN D IN IMMOBILISATION: PART B- MODULATION OF MUSCLE FUNCTIONAL, VASCULAR AND ACTIVATION PROFILES

E.L. BOSTOCK, C.I. MORSE, K. WINWOOD, I.M. MCEWAN, G. L ONAMBÉLÉ

Health, Exercise and Active Living Research Centre, Department of Exercise & Sport Science, Manchester Metropolitan University, Crewe Green Road, Crewe, United Kingdom.
Corresponding author: Gladys Onambele-Pearson, Health, Exercise and Active Living Research Centre, Department of Exercise & Sport Science, Manchester Metropolitan University, Crewe Green Road, Crewe, CW1 5DU, United Kingdom. Tel: +44 (0) 161 247 5394; Email: g.pearson@mmu.ac.uk

Abstract: *Objectives:* This study set out to determine whether two potential protein-sparing modulators (eicosapentaenoic acid and vitamin D) would modulate the anticipated muscle functional and related blood vessels function deleterious effects of immobilisation. *Design:* The study used a randomised, double-blind, placebo-controlled design. *Setting:* The study took part in a laboratory setting. *Participants:* Twenty-four male and female healthy participants, aged 23.0±5.8 years. *Intervention:* The non-dominant arm was immobilised in a sling for a period of nine waking hours a day over two continuous weeks. Participants were randomly assigned to one of three groups: placebo (n=8, Lecithin, 2400 mg daily), omega-3 (ω-3) fatty acids (n=8, eicosapentaenoic acid (EPA); 1770 mg, and docosahexaenoic acid (DHA); 390 mg, daily) or vitamin D (n=8, 1,000 IU daily). *Measurements:* Isometric and isokinetic torque, antagonist muscle co-contraction (activation profile), muscle fatigability indices, and arterial resting blood flow were measured before, at the end of the immobilisation period, and two weeks after re-mobilisation. *Results:* Muscle elbow flexion and extension isometric and isokinetic torque decreased significantly with limb immobilisation in the placebo group ($P<0.05$). Despite no significant effect of supplementation, ω-3 and vitamin D supplementation showed trends ($P>0.05$) towards attenuating the decreases observed in the placebo group. There was no significant change in muscle fatigue parameters or co-contraction values with immobilisation and no effect of supplementation group ($P>0.05$). Similarly, this immobilisation model had no impact on the assessed blood flow characteristics. All parameters had returned to baseline values at the re-mobilisation phase of the study. *Conclusion:* Overall, at the current doses, neither ω-3 nor vitamin D supplementation significantly attenuated declines in torque associated with immobilisation. It would appear that muscle function (described here in Part B) might not be as useful a marker of the effectiveness of a supplement against the impact of immobilisation compared to tissue composition changes (described in Part A).

Key words: Eicosapentaenoic acid, vitamin D, immobilisation, EMG, fatigability.

Introduction

Skeletal muscle adapts to environmental changes and differing levels of physical activity. Periods of disuse, where prolonged reductions in muscle activity and mechanical loading occur, result in profound changes in skeletal muscle morphology and strength in addition to bone parameters. It has consistently been demonstrated, that disuse models, including immobilisation, bed-rest and limb-suspension result in skeletal muscle atrophy (1-3), a decrease in maximal voluntary strength (1-3), and changes in electromyographic characteristics (4). Numerous studies have also documented the effects of immobilisation models on muscle fatigability, with equivocal findings of both decreased, increased and no change in resistance to fatigue (3, 5, 6). With progressing fatigue, there is a shift in electromyography (EMG) to lower frequencies, and median power frequency can be used as an indices of this frequency shift. A decrease in the median power frequency serves as an index of fatigue (7, 8). Fast Fourier transform (FFT) of EMG produces a discrete-time, discrete-frequency representation and can be used to determine median power frequency. There are also several reports of the impact of disuse on the cardiovascular system with reports of decreased reactive hyperaemic blood flow (9-11). Early research demonstrated that static muscular contractions were accompanied by a marked impairment in blood flow to exercising muscles (12). Since then impaired blood flow has been used as an explanation for muscle fatigue during isometric contractions. Local changes in vascular dimensions and blood flow characteristics have been shown in response to resistance training, with de-training resulting in a worsening in theses parameters (beyond pre-training values) (13). Therefore, decreased loading with disuse, may negatively impact on vascular structure and function.

The ever-increasing population of frail elderly puts a strain on healthcare services. Indeed, prolonged sedentarism/ hypo-activity as may be encountered in enforced bed rest, immobilisation owing to orthopaedic clinic events, or simply even, decreased habitual physical activity, all show a high incidence in older persons (14-18). A common and serious problem for older adults is falls, with polypharmacy and some medications contributing to falls in many patients (19). This factor however, is remediable and non-pharmacological interventions are needed to prevent the age-associated loss in muscle size and function. Exercise could be beneficial in these

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circumstances but is not always a practical prescription; and as such, nutritional interventions could be key.

Vitamin D is required to absorb calcium and phosphorus in the body and has been shown to have a direct effect on muscle [20]. Vitamin D plays a vital role in bone maintenance, muscle function, neuromuscular and immune functions, modulation of cell growth and reduction of inflammation. The main source of vitamin D is from sunlight on the skin, with smaller amounts consumed in certain foods. Another way of making sure that the Recommended Dietary Allowance for vitamin D of 600 IU/day (21) is met, is through vitamin D supplementation. Research has demonstrated that vitamin D impacts on both the trans-membranous flows of calcium and phosphate in skeletal muscle, and the synthesis rate of contractile properties (22). Furthermore, research has indicated an association between genetic variation in the vitamin D receptor gene and muscle strength, fat mass and body mass in premenopausal women (23). Vitamin D supplementation in frail elderly women with vitamin D deficiency reduced falls by 49% and improved musculoskeletal function (24). It remains to be seen, in a healthy population with no known vitamin D deficiency, whether vitamin D supplementation would positively affect the response of muscle structural and contractile properties to immobilisation.

Another non-pharmacological agent that may help attenuate the changes associated with immobilisation is Eicosapentaenoic acid (EPA). EPA is an n-3 polyunsaturated fatty acid with anti-inflammatory properties, which is synthesised from ingested alpha-linolenic acid or consumed in fish or in fish oil. Adequate intake (AI) for EPA is set at 1.6 and 1.1 g/day for men and women, respectively (25). Magee et al. suggested that EPA might reduce the pro-inflammatory cytokines associated with inflammation (26). They demonstrated in vitro that EPA inhibits the effects of TNF-α by reducing its apoptotic effects and enabling myogenesis (26). Previous research has suggested the potential of EPA to increase isometric and isokinetic torque (27). It is unclear whether this supplement would have a beneficial effect during immobilisation, where it is generally accepted that there is muscle atrophy (28), which is associated with decreased protein synthesis (29), but scant evidence for increased protein breakdown (30).

As described in Part A (i.e. our previous study), an arm immobilisation model was chosen as it is relatively less restraining on daily life and causes less burden on participants. In Part A, whilst there was no significant effect of supplement group on muscle size decreases with immobilisation, a non-significant trend for lesser atrophy in the treatment groups was seen. Based on the greater decline in muscle strength (due to the combined effects of neural and muscle components) with disuse, it is possible that functional measures are more sensitive to immobilisation than structural changes. Thus, it is possible, that EPA and vitamin D may preserve muscle function to a greater extent than a placebo over the period of immobilisation. The research question, therefore, was: what role, if any, have vitamin D or EPA supplementation in attenuating the changes associated with limb immobilisation? To answer this research question, isometric and isokinetic torque, agonist co-contraction, muscle fatigability and resting arterial blood flow markers, were systematically monitored, immediately post-immobilisation and post-remobilisation, to compare against status pre-immobilisation. Study participants received either ω-3 (a fish oil of a complex of EPA and docosahexaenoic acid (DHA)), vitamin D or a placebo (Lecithin). Hereafter, these agents are simply referred to as EPA, vitamin D or placebo supplementation. It was hypothesised that muscle function will decrease, muscle co-contraction characteristics will worsen, and indices of healthy vascular function will deteriorate, with limb immobilisation. It was also hypothesised that EPA would be the most successful supplement at minimising these changes, as it acts on the protein synthesis pathways.

Methods

Participants & Study Design

Participant inclusion and criteria were as described in study Part A. Briefly, twenty-four healthy volunteers participated in the study, following appropriate ethical approval, and then randomly assigned to one of three groups (PLA: n = 8 (6 females, 2 males); EPA: n = 8 (4 females, 4 males); Vit-D: n = 8 (5 females, 3 males)). The study used a randomised, double-blind, placebo-controlled design with the placebo group consuming 1464 mg Soya Lecithin (Holland & Barrett, UK) daily, the Vit-D group consuming 1,000 IU of Vitamin D3 (Now Foods Bloomingdale, U.S.A.) daily, and the EPA group consuming 1770 mg EPA plus 390 mg docosahexenoic acid (MorEPA, Minami Nutrition, UK), daily.

Participants attended a familiarisation session at least one week prior to the first testing session. After baseline testing, the non-dominant arm was immobilised in a sling for a minimum of nine waking hours a day for two continuous weeks. The correct sling wearing procedure was demonstrated to each participant (Figure 1 of Part A), the removal of the sling was only permitted when necessary (e.g. driving, taking a bath/shower etc.). The sling minimised any movement medio-laterally at the elbow and shoulder and participants were required to not contract the upper musculature (including the hands) during immobilisation hours. Measures of isometric and isokinetic elbow torque, EMG co-contraction, muscle fatigability and arterial dimensions and blood flow, were taken immediately before the immobilisation period (Pre), immediately after the immobilisation period (Post), and two weeks after remobilisation (Post2). During the immobilisation period participants completed a 3-day food diary, a daily activity log (including sling-wear hours) and wore a pedometer (Omron Walking style III step counter, Omron Healthcare Co., Ltd, Kyoto, Japan) to record the number of steps taken each day. The food diaries were analysed for macronutrient

and micronutrient average intake using Microdiet Plus 1.2 (Microdiet, Downlee Systems Ltd, UK). Nutritional information and steps taken each day are reported in the results section of Part A.

Dynamometry

Isometric and isokinetic elbow torque were assessed using a Cybex dynamometer (Cybex, New York, USA). Participants were positioned as per the manufacturer's recommendations. Briefly, they were positioned in a supine position with the axis of rotation of the dynamometer aligned with the anatomical axis of rotation of the elbow joint (lateral epicondyle).

Isometric dynamometry

Following a warm up at 60°/sec at the participant's self-perceived ~75% of maximum effort, two repetitions of isometric contractions were performed at six different elbow joint angles (60°, 70°, 80°, 90°, 100° and 110°), 60 seconds apart. Participants were instructed to rapidly exert maximal torque against the dynamometer lever arm over a 3-4 second period. First in flexion and, five seconds after return to baseline, in extension. Torque and angle were displayed on the screen of a computer (Macintosh G4; Apple Computer, Cupertino, CA), which was interfaced with an A/D system (Acknowledge, Biopac Systems, Santa Barbara, CA) with a sample frequency of 200 Hz. Participants were encouraged to exert maximal torque with the use of visual and verbal feedback. Peak torque was averaged over a 500 ms period (i.e. 250 ms either side of the instantaneous peak). The highest of the repeated efforts was used as the participant's measure of MVC at each angle for elbow flexion and extension.

Isokinetic dynamometry

Isokinetic contractions were completed with three continuous elbow extensions and flexions at six different speeds (30, 60, 90, 120, 180 and 240°/sec) separated by 90 seconds, in a randomised order. The highest of the three consecutive efforts was recorded as peak torque (25 ms either side of the instantaneous peak) for elbow extension and flexion at each speed.

Electromyographic measurements

Muscle activation patterns were assessed using EMG during the isometric and isokinetic contractions. The skin was prepared by shaving, abrading and cleaning with an alcohol-wipe to minimise resistance below 5 kΩ (31). Self-adhesive electrodes were placed in pairs either side of the marker of a third of the distance along the biceps and triceps brachii, with reference electrodes placed on the lateral and medial epicondyle of the humerus. Raw EMG data were recorded at 2000 Hz, with a band pass filter set at 10-500 Hz, and a notch set at 50 Hz (Biopac Systems). Biceps co-contraction was calculated (biceps EMG during extension / biceps EMG during flexion) for both isometric and isokinetic contractions. Triceps co-contraction was also calculated (triceps EMG during flexion / triceps EMG during extension) again for both isometric and isokinetic contractions. Biceps and triceps EMG values were taken during the same windows as in isometric and isokinetic torque.

Fatiguing contractions

The dynamometer lever arm was locked at a 90° angle (where 180° is full elbow extension) and participants were required to exert a maximal isometric contraction in the direction of elbow flexion for 30 seconds. After 90 seconds of recovery, the participant then repeated the maximal isometric contraction for 30 seconds, this time in the direction of elbow extension. The mean, slope and standard deviation of the torque trace were recorded for the length of the 30-second contractions. FFT was computed for the agonist muscle during the first five and last five seconds of each fatiguing contraction. Median frequency values were determined for these time points and a change in median frequency was then computed as a measure of the fatigability of the muscle.

Arterial resting blood flow

After more than 20 minutes seated rest (following muscle ultrasound scans), allowing for the regulation of vascular tone, measurements in the sagittal plane of resting brachial artery diameter, heart rate (HR), resistance index (RI) and flow by diameter (FbD) were obtained from the ultrasound software. The measurements were obtained using an echo Doppler ultrasound machine (AU5, Esaote, Genoa, Italy) with a 5.0- 13.0 MHz broadband linear array transducer (with settings of Doppler gain 37-41, angle of insonation 60 degrees). The ultrasound probe was applied to the arm in line with the marker of the midpoint of the biceps brachii (as previously marked earlier for obtaining muscle ultrasound images). An average of nine cardiac cycles were acquired for all measurements, with the mean value reported.

Statistics

Data were analysed using IBM SPSS v21 (IBM Inc, USA). The Shapiro-Wilk test revealed some of the data to be non-parametric (EMG, fatigue, brachial artery diameter, HR and FbD values). The effect of immobilisation was examined by assessing the changes seen in the PLA group by either repeated measures ANOVA (parametric data) or a Friedman test (non-parametric data). Parametric percentage change values (Pre-to-Post: (Post-Pre)/Pre; and Pre-to-Post2: (Post2-Pre)/Pre) were analysed using a repeated measures ANOVA, with post-hoc Bonferonni corrected 2-tailed t-tests to determine group difference. Non-parametric between group effect were analysed using the Kruskal Wallis test, with post-hoc the Mann-Whitney U tests. All data are presented as mean ± standard deviation (SD). Statistical significance was set with alpha at ≤ 0.05.

Results

Table 1
Fatigue characteristics in response to immobilisation and supplementation

		PLA	EPA	Vit-D
Flexion				
Mean Torque (Nm)	Pre	26.2 ± 12.7	27.1 ± 11.3	28.4 ± 10.8
	Post	24.5 ± 12.1	23.6 ± 10.0	26.8 ± 10.9
	Post2	25.8 ± 12.1	24.7 ± 8.9	26.8 ± 11.4
Flexion				
Standard Deviation	Pre	3.2 ± 1.4	3.4 ± 1.3	3.0 ± 1.3
	Post	2.7 ± 0.8	2.9 ± 1.4	3.5 ± 1.7
	Post2	3.3 ± 1.3	2.7 ± 1.3	3.5 ± 1.7
Flexion				
Slope	Pre	-0.3 ± 0.2	-0.2 ± 0.1	-0.2 ± 0.1
	Post	-0.3 ± 0.2	-0.2 ± 0.1	-0.3 ± 0.2
	Post2	-0.3 ± 0.2	-0.1 ± 0.1	-0.1 ± 0.1
Flexion				
Agonist FFT				
1st Five Seconds (Hz)	Pre	113.6 ± 22.6	102.9 ± 8.5	102.3 ± 5.3
	Post	116.5 ± 13.5	106.5 ± 7.8	103.9 ± 5.1
	Post2	112.1 ± 17.3	103.7 ± 8.0	102.1 ± 4.7
Flexion				
Agonist FFT				
Last Five Seconds (Hz)	Pre	115.2 ± 16.7	102.1 ± 8.0	104.2 ± 9.5
	Post	119.1 ± 15.8	105.5 ± 5.3	105.6 ± 8.5
	Post2	113.6 ± 12.7	101.8 ± 8.3	104.2 ± 9.2
Extension				
Mean Torque (Nm)	Pre	20.4 ± 14.4	23.8 ± 11.8	21.9 ± 12.1
	Post	19.2 ± 13.5	19.2 ± 8.7	17.7 ± 10.2
	Post2	21.3 ± 12.1	22.5 ± 10.7	22.3 ± 9.6
Extension				
Standard Deviation	Pre	2.9 ± 1.9	2.4 ± 1.6	2.2 ± 1.3
	Post	2.5 ± 1.5	2.5 ± 1.4	2.1 ± 0.9
	Post2	2.8 ± 2.1	2.2 ± 1.4	2.1 ± 1.1
Extension				
Slope	Pre	-0.3 ± 0.6	0.1 ± 0.1	-0.2 ± 0.3
	Post	-0.2 ± 0.2	-0.1 ± 0.2	-0.2 ± 0.2
	Post2	-0.3 ± 0.4	-0.1 ± 0.3	-0.1 ± 0.1
Flexion				
Agonist FFT				
1st Five Seconds (Hz)	Pre	131.8 ± 13.6	128.9 ± 13.3	131.5 ± 9.7
	Post	132.6 ± 16.5	130.3 ± 12.0	133.2 ± 13.2
	Post2	142.3 ± 15.8	130.1 ± 14.4	131.7 ± 9.9
Flexion				
Agonist FFT				
Last Five Seconds (Hz)	Pre	124.7 ± 17.0	124.1 ± 16.0	127.7 ± 12.4
	Post	125.0 ± 19.3	125.7 ± 15.3	129.4 ± 13.7
	Post2	124.3 ± 18.6	125.1 ± 15.9	128.5 ± 12.4

Mean values ± SD for fatigue characteristics. The response of both the torque trace and fast Fourier transform of the agonist muscle during both a 30 second elbow flexion maximal fatiguing contraction and a 30 second elbow extension fatiguing contraction.

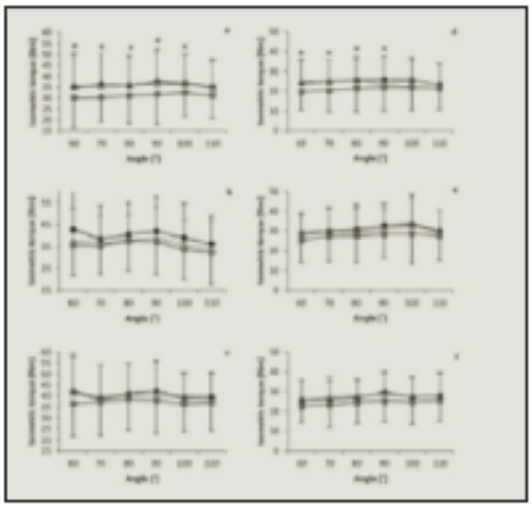
Baseline characteristics

There were no significant differences in baseline characteristics (Table 1 of series A). Additionally, the groups did not differ in baseline isometric MVC elbow torque (e.g. isometric elbow torque at 90° for flexion - PLA: 37.6 ± 15.0 Nm; EPA: 42.0 ± 15.7 Nm; Vit-D: 42.3 ± 13.7 Nm) EMG and fatigue values, or resting arterial blood vessel characteristics (vessel diameter - PLA: 3.3 ± 0.5 mm; EPA: 3.3 ± 0.6 mm; Vit-D: 3.2 ± 0.3 mm, HR – PLA: 69.6 ± 11.6 bpm; EPA: 68.5 ± 12.1 bpm; Vit-D: 69.1 ± 6.7 bpm, RI – PLA: 0.7 ± 0.3; EPA: 0.8 ± 0.1; Vit-D: 0.9 ± 0.1, FbD – PLA: 0.11 ± 0.05 m/s; EPA: 0.07 ± 0.02 m/s; Vit-D: 0.06 ± 0.03 m/s).

Isometric dynamometry

Isometric MVC torque decreased for both elbow flexion and extension at every angle ($p<0.05$) except for flexion at 110° and extension at 100° and 110°. Average isometric torque decrease from Pre to Post immobilisation across angles for flexion were 12.1 ± 1.8 %, 11.4 ± 3.5 % and 8.1 ± 3.4 %, and for extension were 15.4 ± 3.3 %, 12.0 ± 3.1 % and 10.7 ± 2.4 %, for PLA, EPA and Vit-D, respectively. There was no effect of group on the percentage change in isometric torque for flexion or extension at any of the six angles (Figure 1).

Figure 1
Isometric torque changes in response to immobilisation and supplementation



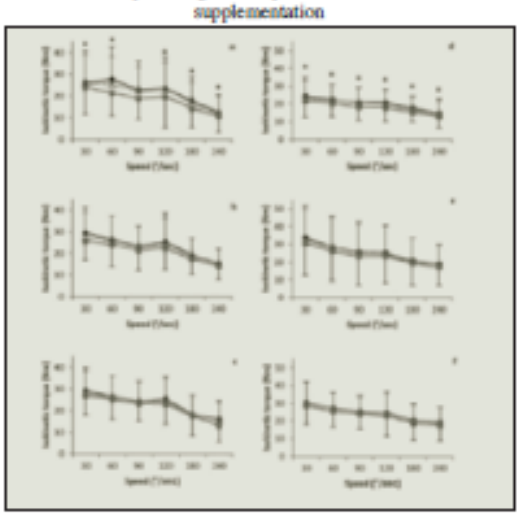
Pre (circles), Post (squares) and Post2 (triangles) values for isometric torque (Nm ± SD) for elbow flexion (a = PLA; b = EPA; c = Vit-D) and elbow extension (d = PLA; e = EPA; f = Vit-D) at the six different angles (60-110°). * Significant difference between Pre and Post immobilisation in the PLA group.

Isokinetic dynamometry

Isokinetic torque decreased significantly for both elbow flexion and extension at every speed ($p<0.05$) except for flexion at 90°/sec. Average isokinetic torque decrease from Pre to Post immobilisation across speeds for flexion were 14.0 ± 4.5 %, 8.6 ± 1.3 % and 7.8 ± 5.5 %, and for extension were 10.3 ± 1.8 %, 8.1 ± 0.9 % and 7.1 ± 1.8 %, for PLA, EPA and Vit-D, respectively. There was no effect of supplement group on the percentage change in isokinetic torque for flexion or extension at any of the six speeds. (Figure 2)

Figure 2

Isokinetic torque changes in response to immobilisation and supplementation



Pre (circles), Post (squares) and Post2 (triangles) values for isokinetic torque (Nm ± SD) for elbow flexion (a = PLA; b = EPA; c = Vit-D) and elbow extension (d = PLA; e = EPA; f = Vit-D) at the six different speeds (30-240°/sec). * Significant difference between Pre and Post immobilisation in the PLA group.

Electromyographic measurements

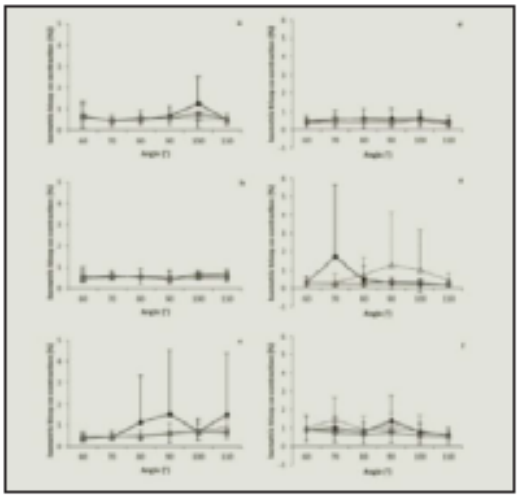
Analysis of biceps and triceps co-contraction RMS EMG values during the isometric contractions showed no significant changes from Pre to Post immobilisation and no effect of supplement group from Pre-to-Post and Pre-to-Post2 (Figure 3). Similarly, this was the case for the isokinetic contractions with no significant effect of immobilisation nor of supplement group on the EMG response to the immobilisation (Figure 4).

Fatiguing contractions

The mean, slope and standard deviation of the torque traces showed no significant changes with immobilisation phase for either flexion or extension fatiguing contractions, with no effect of supplement group on percentage changes. Analysis of the fast Fourier transformation of EMG traces of the biceps and triceps brachii revealed no significant effect of immobilisation or supplementation group on rate coding at either the beginning, or the end of a fatiguing maximal isometric contraction (Table 1).

Figure 3

Isometric bicep and triceps co-contraction changes in response to immobilisation and supplementation



Pre (circles), Post (squares) and Post2 (triangles) values for co-contraction (Nm ± SD) of the biceps (a = PLA; b = EPA; c = Vit-D) and triceps (d = PLA; e = EPA; f = Vit-D) during isometric elbow flexion and extension at the six different angles (60-110°).

Arterial resting blood flow

Brachial artery diameter, RI, FbD and HR did not change from Pre to Post immobilisation, nor Pre to Post2. There was no significant difference in the percentage change in brachial artery diameter, RI, FbD or HR between the groups (Table 2).

Measurement reliability

All protocols were assessed for intra as well as inter-day reliability. This utilised five participants and entailed carrying out measurements three times on day 1, and repeating these on day 2, approximately a week later. Within-day coefficients of variation (CV) of 1.8 %, 2.0 %, 1.5 %, 5.4 %, 2.4 % and 12.0 %, and between-day CVs of 2.5 %, 2.3 %, 2.3 %, 8.1 %, 4.0 % and 9.1 % were yielded for isometric torque, isokinetic torque, brachial artery diameter, HR, RI and FbD, respectively.

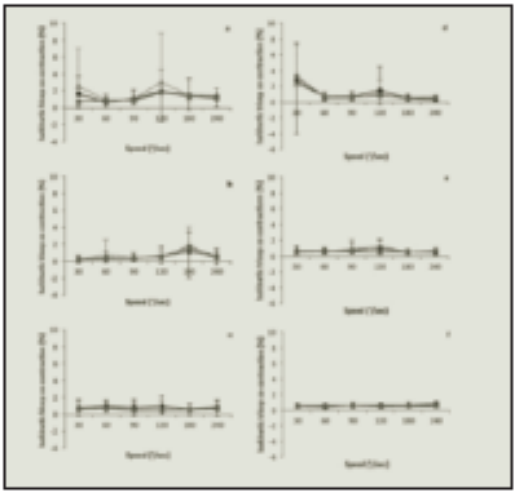
Discussion

We describe the effects of two potential protein-sparing modulators (EPA of vitamin D supplementations) on the response to 2 weeks of 9-waking-hours-per-day combined arm and shoulder immobilisation. We hypothesised that

muscle function will decrease and muscle co-contraction characteristics will increase, and indices of healthy vascular function will deteriorate, with limb immobilisation. We found evidence to partially support our hypotheses, with significant immobilisation-induced decreases in isometric elbow flexion (PLA: 6.7 to 18.4%) and extension (PLA: 8.7 to 13.8%) torque, as well as isokinetic elbow flexion (PLA: 9.3 to 13.7%) and extension (PLA: 9.8 to 18.1%) torque. It was also hypothesised that EPA would be the most effective supplement at minimising the response to immobilisation. Our data demonstrate that neither EPA nor vitamin D had any significant effect on the responses to non-injurious immobilisation. Nonetheless, a few trends towards the attenuation of elbow isometric and isokinetic torque immobilisation-induced decreases were observed, in the EPA and Vit-D treated groups. We discuss the observed trends for the attenuation of these parameters. It is also notable that this immobilisation model had no impact on the assessed co-contraction and muscle fatigability, or on the assessed blood flow characteristics.

Figure 4

Isokinetic bicep and triceps co-contraction changes in response to immobilisation and supplementation



Pre (circles), Post (squares) and Post2 (triangles) values for co-contraction (Vm ± SD) of the biceps (a = PLA; b = EPA; c = Vit-D) and triceps (d = PLA; e = EPA; f = Vit-D) during isokinetic elbow flexion and extension at the six different speeds (30-240°/sec).

Isometric elbow extensor and flexor torque decreased following immobilisation. This supports previous findings of decreases in isometric MVC of the elbow flexors in response to 4 weeks of elbow cast immobilisation (6). In addition to the established decline in isometric torque, disuse models also result in reductions in dynamic torque outputs. Cast immobilisation of the arm (9 days) also results in decreased concentric and eccentric strength for flexion and extension of the wrist (3). Our current data also show a decrease in isokinetic strength for both elbow flexion and extension. However, muscle function in terms of isometric and/or isokinetic torque, did not show a significant effect of either EPA or vitamin D supplementation at the current doses, in spite of somewhat blunting the effect of immobilisation, as this protective effect was not statistically significant.

Data collected for agonist and antagonist EMG activity highlighted no differences in biceps or triceps co-contraction following immobilisation. In contrasting, some of the previous research shows a large decrease in EMG amplitude measurements during flexion in both the agonist and antagonist muscle (4, 6). When drawing conclusions from EMG findings care should be taken, as: 1) the changes in muscle dimensions could result in a different population of motor units being recorded from (32); 2) EMG reliability in previously published studies is not very high, and this is a general limitation of studies utilising longitudinal EMG monitoring (33, 34). In addition, it is notable that EMG data in our, and previous work (31), does not normalise the data for the clarity of the signal. Specifically, we have recently demonstrated that sub-cutaneous adiposity changes with immobilisation (see study Part A of our current work), hence the electromyographic signature would have differed (35).

Numerous studies have documented the effects of immobilisation models on muscle fatigability, with equivocal findings of both decreased, increased and no change in resistance to fatigue (3, 5, 6). In our current study, we found no significant changes in the mean, slope or standard deviation of the torque trace during the 30s isometric contraction fatigue tasks that we implemented. Differences between studies could be due to the mode/duration of disuse or in the method used to test fatigue resistance since fibre recruitment level and pattern would vary with changes in contractile profile (36). The mechanisms behind the varying effects of immobilisation on muscle fatigability are yet to be fully explained. Our observation of no significant effect of immobilisation on the fast Fourier transformation of EMG traces at either the beginning or end of a maximal isometric contraction suggests no effect on motor unit rate coding and none on fibre type recruitment.

Previous research suggests that decreases in physical activity leads to detrimental vascular adaptations (37, 38). Bed-rest studies measuring leg blood flow report inconsistent results, with some showing no changes in leg blood flow and others demonstrating a decrease in leg blood flow after periods of bed rest (10-41 days) (38-41). None of these bed rest studies report data on changes in arterial vessel diameter. Studies investigating vascular changes in response to upper limb immobilisation are lacking. We report no significant dimensional changes in vasculature with upper limb immobilisation. Similarly, we observed no significant changes in resting HR in response to immobilisation. Equally,

Table 2
Changes in blood kinetic parameters in response to immobilisation and supplementation

	PLA		EPA		Vit-D	
	Pre-to-Post	Pre-to-Post2	Pre-to-Post	Pre-to-Post2	Pre-to-Post	Pre-to-Post2
Brachial Artery Diameter	1.0 ± 14.7	3.7 ± 10.4	2.0 ± 10.8	2.4 ± 9.4	0.7 ± 8.2	3.6 ± 6.5
Resistance Index (RI)	-4.6 ± 30.6	1.2 ± 20.7	-5.2 ± 15.1	-8.3 ± 9.1	-0.9 ± 15.1	-0.9 ± 15.1
Flow by Diameter (FbD)	-14.6 ± 31.4	-8.8 ± 50.6	-7.3 ± 8.7	-12.9 ± 9.0	-9.5 ± 34.5	29.5 ± 15.5
Heart Rate (HR)	-3.5 ± 11.0	-2.4 ± 6.9	4.0 ± 12.1	6.1 ± 15.7	4.3 ± 16.3	2.3 ± 14.9

Percentage change (% ± SD) in blood kinetic parameters from Pre-to-Post and Pre-to-Post2 for PLA, EPA and Vit-D. * Significant difference between Pre and Post immobilisation in the PLA group. † Significant difference in % change between groups.

our assessment of resting arterial blood flow (diameter, RI and FbD) revealed no significant changes in response to immobilisation; however, we report resting and not reactive blood flow and as such were less likely to see any effect (11). The lack of change in muscle fatigability, however, goes hand in hand with the absence of vascularisation-related alterations. Recently, higher circulating concentrations of 1,25-dihydroxyvitamin D have been associated with a higher risk of hypertension (42). The authors suggested that the vitamin D-induced increase in calcium absorption could promote vascular calcification, which in turn could lead to increased arterial stiffness and greater hypertension risk. In the current study, vitamin D supplementation did not impact on the measured resting arterial blood flow parameters, however, in the current study this was a short duration supplementation that may not have resulted in comparable increases in circulating concentrations of 1,25-dihydroxyvitamin D. The lack of impact of EPA supplementation on the measured resting arterial blood flow parameters in the current study, concurs with recent findings, that higher serum biomarkers of ω-3 consumption, may not impact on future blood pressure (43).

Conclusion

In summary, upper limb immobilisation resulted in a decrease in elbow isometric and isokinetic torque, with no observed effect on co-contraction, muscle fatigability or resting blood flow. We observed no significant effect of EPA or vitamin D supplementation on any of these parameters. Despite greater relative decreases in torque than in tissue composition (Part A), there is no significant effect of EPA or vitamin D supplementation on the decreases in torque. It would appear that muscle function might be a less sensitive marker of the effectiveness of a supplement against the impact of immobilisation than tissue composition.

Ethics declaration: All experimental procedures were conducted in accordance with the guidelines in the Declaration of Helsinki and approved by the Ethics Committee of Manchester Metropolitan University.

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Conflict of Interest Disclosure: The authors do not have any conflicts of interest.

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Appendix 2: Conference proceedings

THE EFFECTS OF 2 WEEKS ARM IMMOBILISATION ON MUSCLE FUNCTION MODULATORS

Bostock E, Morse CI, Winwood K, McEwan I, Onambélé-Pearson G.
MMU (Manchester, UK)

Introduction: Prolonged reduction in muscle activity and mechanical loading, as seen with limb immobilisation, results in skeletal muscle structural and functional maladaptation including atrophy and asthenia (see review by Bostock et al., 2013). Previous research suggests that immobilisation results in a greater decrease in muscle strength than muscle size which may be a result of neuromuscular adaptations (Miles et al., 1994). The aim of this study was, thus, to investigate the principal physiological contributors to asthenia induced through upper limb immobilisation.

Methods: The non-dominant arm of 6 healthy, habitually active females (aged 29 ± 6 years) was immobilised in a sling for nine waking hours a day for two continuous weeks. Measures of tissue thickness (B-mode ultrasonography), arm girth (anthropometry), body composition (DEXA), isometric (60-110° elbow angle) and isokinetic (30-240°/s) torque (dynamometry), vascular kinetics (Doppler ultrasonography), fatigue index (slope of electromyography trace) and Creatine Kinase Activity (Colorimetry at 340nm) were taken before immobilisation (PRE), on removal of the sling (POST), and two weeks after re-mobilisation (POST2).

Results: No differences existed between the immobilised and non-immobilised limbs at baseline for any measures ($p>0.05$). PRE to POST changes in the immobilised limb in arm girth (upper = -1.2%, lower = -1.0%), muscle thickness (biceps = -3.5 to -6.5%, triceps = -7.1 to -10.2%), lean mass (-4.7%), sub-cutaneous adipose thickness (7.7 to 12.7%) and bone mineral content (-2.7%) were significantly different from those in the non-immobilised limb ($p<0.05$). These changes reverted to PRE values at POST2 (except for adipose thickness and bone mineral content). Torque significantly decreased with immobilisation (-5.2 to -17.5% changes, $p<0.05$), with only one torque test not reverting to PRE values at POST2. There was a trend towards an increase in Creatine Kinase activity at POST ($p>0.05$). Immobilisation did not affect muscle fatigability or vascular kinetics.

Discussion: Our data demonstrate that a relatively modest degree of limb immobilisation is sufficient to impact on muscle, adipose, and bone parameters. Whilst our results support the previously observed dissociation between degree of atrophy and asthenia (Miles et al., 1994), the physiological modulator of this effect remains unclear. Indeed, neither blood flow, vascular dimensions nor compliance, nor muscle fatigability, changed in this cohort. We propose that endocrine profile changes may be key to the early response to immobilisation.

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Two nutrition supplements modulate aspects of immobilisation-induced changes in appendicular mass characteristics

Emma L. Bostock[1], Christopher I. Morse[1], Keith Winwood[1], Islay McEwan[1] and Gladys L. Onambélé[1]

[1]Centre for Health, Exercise and Active Living, Department of Exercise & Sport Science, Manchester Metropolitan University, Crewe, Cheshire, United Kingdom.

Background: Muscle size and strength decrease in response to short-term limb immobilisation [1]. Where exercise prescription is not possible (e.g. bed rest or injury) other interventions are required to attenuate the atrophy and strength declines associated with disuse. There is a necessity to identify non-pharmacological interventions since polypharmacy in itself is conducive to skeletal tissue loss [2].

Objectives: To determine whether two potential protein-sparing modulators (Eicosapentaenoic acid (EPA) and Vitamin D (Vit-D)) would attenuate immobilisation-induced changes in muscle characteristics.

Methods: The non-dominant arm of n=24 healthy participants, aged 23 ± 5.8 years, was immobilised daily in a sling for a period of 9 waking hours over two continuous weeks. Participants were randomly assigned to one of three groups: placebo (Lecithin), EPA or Vit-D. Muscle and sub-cutaneous adipose thickness (B-mode ultrasonography), blood flow kinetics (colour Doppler ultrasound), isometric and isokinetic torque (dynamometry) were measured immediately before, immediately after the immobilisation period and two weeks after re-mobilisation.

Results: There was a significant decrease in muscle thickness (-5.4%), isometric torque (flexion: -12.1%, extension: -15.4%) and isokinetic torque (flexion: -14.0%, extension: -10.3%) with limb immobilisation in the placebo group ($P<0.05$). Despite no significant effect of group, EPA and Vit-D supplementation showed trends towards attenuating the decreases in muscle thickness and isometric and isokinetic torque observed in the placebo group. Sub-cutaneous adipose thickness increased in the placebo group (10.3%). EPA ($p<0.05$) and Vit-D ($p>0.05$) blunted this response, with EPA having a greater effect ($p<0.05$). Immobilisation had no significant effect on blood flow kinetics, including heart rate, brachial artery diameter, resistance index and blood flow by vessel diameter.

Conclusions: Both EPA and Vit-D supplementation generally attenuated the changes associated with immobilisation. These findings may be applicable to both sporting (e.g. off-season detraining modulation) and clinical (injury/surgery induced short-term immobilisation) populations.

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Keywords: Eicosapentaenoic Acid; Vitamin D; Body Composition; Immobilisation; Lecithin.

Modulation of tissue composition and function by eicosapentaenoic acid and vitamin D in immobilisation

Emma L. Bostock[1], Christopher I. Morse[1], Keith Winwood[1], Islay McEwan[1] and Gladys L. Onambélé[1]
[1]Department of Exercise & Sport Science, Manchester Metropolitan University, Crewe, United Kingdom.

It has consistently been demonstrated that disuse models (e.g. limb immobilisation and bed rest), result in skeletal muscle atrophy, decreases in maximal voluntary strength (1) and bone mineral density (2), and increases in intermuscular adipose content (3). Disuse models have also been associated with changes in electromyography (EMG) characteristics (4) and muscle fatigability (1). Where exercise prescription is not possible (e.g. bed rest or injury), other interventions are required to attenuate these changes associated with disuse. Non-pharmacological interventions need to be identified since polypharmacy in itself is conducive to skeletal tissue loss (5). This study set out to determine whether two potential protein-sparing modulators (eicosapentaenoic acid [EPA] and vitamin D [VitD]) would modulate the deleterious effects of immobilisation. The non-dominant arm of 24 healthy participants, aged 23.0±5.8 years, was immobilised in a sling for a period of 9 waking hours a day over two continuous weeks. Participants were randomly assigned to one of three groups: placebo (PLA) (n=8), EPA (n=8) or VitD (n=8). Body composition (DEXA), arm girth (anthropometry), muscle co-contraction (EMG) and muscle fatigability (Cybex and EMG) were measured before, at the end of the immobilisation period and two weeks after re-mobilisation. The effect of immobilisation and supplement group were assessed by either repeated measures ANOVA (parametric data), with post-hoc Bonferonni corrected 2-tailed t-tests or Kruskal Wallis test (non-parametric data), with post-hoc Mann-Whitney U tests. All data are presented as mean ± standard deviation. There were significant decreases in upper and lower arm girth, lean mass and bone mineral content (BMC) post-immobilisation in the PLA group ($p<0.05$). Despite no significant effect of group, EPA and VitD supplementation showed trends towards attenuating the decreases in upper/lower arm girths (-1.3±0.4% PLA, -0.6±0.5% EPA, -0.9±1.0% VitD, $p=0.18$ and -0.8±0.8% PLA, -0.4±0.5% EPA, -0.3±0.4% VitD, $p=0.21$, respectively) and BMC (-2.3±1.5% PLA, -0.3±1.0% EPA, -0.7±1.9% VitD, $p=0.47$) observed in the PLA group. The EPA supplementation group demonstrated a non-significant attenuation of the decrease in lean mass observed in the placebo group (-3.6±3.7% PLA, -1.9±2.8% EPA, -4.0±2.8% VitD, $p=0.95$). There was no significant change in muscle fatigue parameters or EMG co-contraction values with immobilisation and no effect of supplementation group ($p>0.05$). All parameters had returned to baseline values at the re-mobilisation phase of the study. The results suggest that both EPA and VitD may generally attenuate the changes in muscle size and bone parameters associated with immobilisation. These findings may be applicable to both sporting (e.g. off-season detraining) and clinical (injury/surgery induced short-term immobilisation) populations.

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Appendix 3: Informed consent form

Department of Exercise and Sport Science

Informed Consent Form

(Both the investigator and participant should retain a copy of this form)

Name of Participant:

Supervisor/Principal Investigator: Emma Bostock

Project Title: Impact of nutritional supplementation on immobilisation-induced atrophy

Ethics Committee Approval Number: 150109(i)

Participant Statement

I have read the participant information sheet for this study and understand what is involved in taking part. Any questions I have about the study, or my participation in it, have been answered to my satisfaction. I understand that I do not have to take part and that I may decide to withdraw from the study at any point without giving a reason. Any concerns I have raised regarding this study have been answered and I understand that any further concerns that arise during the time of the study will be addressed by the investigator. I therefore agree to participate in the study.

It has been made clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the Registrar and Clerk to the Board of Governors, Head of Governance and Secretariat Team, Manchester Metropolitan University, All Saints Building, All Saints, Manchester, M15 6BH, Tel: 0161 247 1390 who will undertake to investigate my complaint.

Signed (Participant)		Date	
Signed (Investigator)		Date	

Appendix 4: Questionnaire

Participant ID code:_____

MMU Cheshire

Department of Exercise and Sport Science

Impact of Nutritional Supplementation on Immobilisation-Induced Atrophy

Questionnaire: Physical Activity & General Health

Thank you for your interest in this research study. Prior to participation, we would like you to answer a few questions concerning your general health and physical activity level. Please answer the following questions as honestly as possible.

Height (m): _____m

Weight (Kg):_____Kg

Date of birth (dd/mm/yy): ___ /___ /___

Sex: Male ☐ Female ☐

How would you best describe your ethnicity?

White – British	☐	Black or Black British – African	☐
Black or Black British – Caribbean	☐	Asian or Asian British – Pakistani	☐
Asian or Asian British – Banglasdeshi	☐	Chinese	☐
Other Asian Background	☐	Other Ethnic Background	☐

If other please specify:_____

Smoking Habits	Are you currently a smoker?	Yes / No
	If yes, how many do you smoke?	……. per day
	Are you a previous smoker?	Yes / No
	If yes, how long is it since you stopped?	…… years

Do you drink alcohol? Yes / No

If you answered Yes, do you usually have:

An occasional drink	☐
A drink every day	☐
More than one drink a day	☐

Your general health

1. **At present,** do you have any health problems for which you are:

a) on medication, prescribed (by a doctor) or otherwise _____ Yes ☐ No ☐
b) attending (visiting) your doctor _____ Yes ☐ No ☐
c) on a hospital waiting list _____ Yes ☐ No ☐

2. **Have you ever** had any of the following?

a) Your doctor advised you not to take vigorous exercise _____ Yes ☐ No ☐
b) Pain in your chest when you undertake physical activity? _____ Yes ☐ No ☐
c) Central Nervous System disease, such as Parkinson, Alzheimer, Convulsions/epilepsy _____ Yes ☐ No ☐
d) Have you any history of chest problems, such as bronchitis, asthma or wheezy chest _____ Yes ☐ No ☐
e) Major illness, such as viral hepatitis, cancer _____ Yes ☐ No ☐
f) Eczema _____ Yes ☐ No ☐
g) Diabetes _____ Yes ☐ No ☐
h) High blood pressure _____ Yes ☐ No ☐
i) A limb fracture _____ Yes ☐ No ☐
j) Blood disorder, such as clotting problems, thrombosis, aneurysm, embolus _____ Yes ☐ No ☐
k) Head injury _____ Yes ☐ No ☐
l) Digestive problems _____ Yes ☐ No ☐
m) Heart problems, such as heart attack, valve disease, palpitations, angina _____ Yes ☐ No ☐
n) Problems with bones, such as osteoporosis or osteoarthritis _____ Yes ☐ No ☐
o) Problems with joints, such as rheumatoid arthritis, any persistent pain, or any surgery on your joints _____ Yes ☐ No ☐
p) Back problems _____ Yes ☐ No ☐
q) Disturbance of balance/co-ordination, such as dizziness or balance- system dysfunction _____ Yes ☐ No ☐
r) Numbness in hands or feet _____ Yes ☐ No ☐
s) Disturbance of vision _____ Yes ☐ No ☐
t) Physical limitations, such as visual, hearing, walking problems _____ Yes ☐ No ☐
u) Thyroid problems, e.g. rapid loss or gain of weight _____ Yes ☐ No ☐
v) Kidney or liver problems _____ Yes ☐ No ☐
w) A severe allergic reaction, e.g. swelling, breathing difficulties in response to an external stimulus _____ Yes ☐ No ☐
x) Emotional or psychiatric problems _____ Yes ☐ No ☐
y) Any other illness or condition that affects your general health or interferes with your daily activities _____ Yes ☐ No ☐

3. If you answered **YES** to any of the questions above, please describe the details briefly below or to the investigator if you wish.

_____

_____

4. Are you currently involved in any other research studies at the University or elsewhere?

Yes ☐ No ☐

If YES please provide details of the study: _____

_____

Habitual physical activity

1. What is your main occupation? _____

2. At work I sit Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Always ☐
3. At work I stand Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Always ☐
4. At work I walk Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Always ☐
5. At work I lift heavy loads Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Always ☐
6. After work I am tired Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Always ☐
7. At work I sweat Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Always ☐
8. In comparison with others my own age I think my work is:
   Much heavier ☐ Heavier ☐ As heavy ☐ Lighter ☐ Much lighter ☐

9. Do you play sport or exercise? Yes ☐ No ☐
   If **YES**, which sport do you play most frequently? _____

How many hours per week?	Less than 1 ☐	1 to 2 ☐	2 to 3 ☐	3 to 4 ☐	More than 4 ☐
Time per session (hours)	½ ☐	1½ ☐	2½ ☐	3½ ☐	4½ ☐
How many months per year?	Less than 1 ☐	1 to 3 ☐	4 to 6 ☐	7 to 9 ☐	More than 9 ☐
What proportion of the month?	A few hours ☐	A few days ☐	2 weeks ☐	3 weeks ☐	Most of the month ☐

If you do a **second** sport (or exercise class), which is it?

How many hours per week?	Less than 1 ☐	1 to 2 ☐	2 to 3 ☐	3 to 4 ☐	More than 4 ☐
Time per session (hours)	½ ☐	1½ ☐	2½ ☐	3½ ☐	4½ ☐
How many months per year?	Less than 1 ☐	1 to 3 ☐	4 to 6 ☐	7 to 9 ☐	More than 9 ☐
What proportion of the month?	A few hours ☐	A few days ☐	2 weeks ☐	3 weeks ☐	Most of the month ☐

10. Compared with other of my own age I think my physical activity during leisure time is:
    Much more ☐ More ☐ The same ☐ Less ☐ Much less ☐
11. During leisure time I sweat Very often ☐ Often ☐ Sometimes ☐ Seldom ☐ Never ☐
12. During leisure time I play sport Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Always ☐
13. During leisure time I watch TV Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Always ☐
14. During leisure time I walk Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Always ☐
15. During leisure time I cycle Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Always ☐
16. How many minutes do you walk per day to and from work, school and/or shopping?
    Less than 5 ☐ 5 to 15 ☐ 16 to 30 ☐ 31 to 45 ☐ More than 45 ☐

Thank you for taking the time to complete this questionnaire. All information will be kept strictly confidential

Appendix 5: Extra statistical analysis

The effect of immobilisation was examined in chapters 3 and 4 by assessing the changes seen in the placebo group by either repeated measures ANOVA (parametric data) or a Friedman test (non-parametric data). The next few pages provide additional statistical analysis, with either repeated measures ANOVA or a Friedman test, on the raw data of the individual groups (placebo (PLA), EPA or vitamin D (Vit-D)) pre-immobilisation (Pre), post-immobilisation (Post) and post remobilisation (Post2).

Chapter 3: PLA group (data used in the text)

	Pre to Post immobilisation	Pre to Post2 immobilisation
Upper arm girth	$p < 0.05$	$p > 0.05$
Lower arm girth	$p < 0.05$	$p > 0.05$
Biceps muscle thickness (L50)	$p < 0.05$	$p > 0.05$
Biceps muscle thickness (L33	$p < 0.05$	$p > 0.05$
Triceps muscle thickness (L50)	$p < 0.05$	$p > 0.05$
Triceps muscle thickness (L33)	$p < 0.05$	$p > 0.05$
Biceps sub-cutaneous adipose thickness (L50)	$p < 0.05$	$p > 0.05$
Biceps sub-cutaneous adipose thickness (L33)	$p > 0.05$	$p > 0.05$
Triceps sub-cutaneous adipose thickness (L50)	$p > 0.05$	$p > 0.05$
Triceps sub-cutaneous adipose thickness (L33)	$p > 0.05$	$p > 0.05$
Lean mass	$p < 0.05$	$p > 0.05$
BMC	$p < 0.05$	$p > 0.05$
BMD	$p > 0.05$	$p > 0.05$
Fat mass	$p > 0.05$	$p > 0.05$
Fat %	$p > 0.05$	$p > 0.05$

Chapter 3: EPA group

	Pre to Post immobilisation	Pre to Post2 immobilisation
Upper arm girth	p < 0.05	p > 0.05
Lower arm girth	p < 0.05	p > 0.05
Biceps muscle thickness (L50)	p < 0.05	p > 0.05
Biceps muscle thickness (L33	p < 0.05	p > 0.05
Triceps muscle thickness (L50)	p < 0.05	p > 0.05
Triceps muscle thickness (L33)	p < 0.05	p > 0.05
Biceps sub-cutaneous adipose thickness (L50)	p > 0.05	p > 0.05
Biceps sub-cutaneous adipose thickness (L33)	p > 0.05	p > 0.05
Triceps sub-cutaneous adipose thickness (L50)	p > 0.05	p > 0.05
Triceps sub-cutaneous adipose thickness (L33)	p > 0.05	p > 0.05
Lean mass	p > 0.05	p > 0.05
BMC	p > 0.05	p > 0.05
BMD	p > 0.05	p > 0.05
Fat mass	p > 0.05	p > 0.05
Fat %	p > 0.05	p > 0.05

Chapter 3: Vit-D group

	Pre to Post immobilisation	Pre to Post2 immobilisation
Upper arm girth	p < 0.05	p > 0.05
Lower arm girth	p < 0.05	p > 0.05
Biceps muscle thickness (L50)	p < 0.05	p > 0.05
Biceps muscle thickness (L33	p < 0.05	p > 0.05
Triceps muscle thickness (L50)	p < 0.05	p > 0.05
Triceps muscle thickness (L33)	p < 0.05	p > 0.05
Biceps sub-cutaneous adipose thickness (L50)	p < 0.05	p > 0.05
Biceps sub-cutaneous adipose thickness (L33)	p < 0.05	p > 0.05
Triceps sub-cutaneous adipose thickness (L50)	p < 0.05	p > 0.05
Triceps sub-cutaneous adipose thickness (L33)	p > 0.05	p > 0.05
Lean mass	p < 0.05	p > 0.05
BMC	p > 0.05	p > 0.05
BMD	p > 0.05	p > 0.05
Fat mass	p > 0.05	p > 0.05
Fat %	p > 0.05	p > 0.05

Chapter 4: PLA group (data used in the text)

	Pre to Post immobilisation	Pre to Post2 immobilisation
Isometric torque flexion (60°)	$p < 0.05$	$p > 0.05$
Isometric torque flexion (70°)	$p < 0.05$	$p > 0.05$
Isometric torque flexion (80°)	$p < 0.05$	$p > 0.05$
Isometric torque flexion (90°)	$p < 0.05$	$p > 0.05$
Isometric torque flexion (100°)	$p < 0.05$	$p > 0.05$
Isometric torque flexion (110°)	$p > 0.05$	$p > 0.05$
Isometric torque extension (60°)	$p < 0.05$	$p > 0.05$
Isometric torque extension (70°)	$p < 0.05$	$p > 0.05$
Isometric torque extension (80°)	$p < 0.05$	$p > 0.05$
Isometric torque extension (90°)	$p < 0.05$	$p > 0.05$
Isometric torque extension (100°)	$p > 0.05$	$p > 0.05$
Isometric torque extension (110°)	$p > 0.05$	$p > 0.05$
Isokinetic torque flexion (30°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (60°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (90°/sec)	$p > 0.05$	$p > 0.05$
Isokinetic torque flexion (120°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (180°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (240°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (30°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (60°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (90°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (120°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (180°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (240°/sec)	$p < 0.05$	$p > 0.05$

	Pre to Post immobilisation	Pre to Post2 immobilisation
Isometric torque flexion (60°)	$p < 0.05$	$p > 0.05$
Isometric torque flexion (70°)	$p > 0.05$	$p > 0.05$
Isometric torque flexion (80°)	$p > 0.05$	$p > 0.05$
Isometric torque flexion (90°)	$p < 0.05$	$p > 0.05$
Isometric torque flexion (100°)	$p < 0.05$	$p > 0.05$
Isometric torque flexion (110°)	$p > 0.05$	$p > 0.05$
Isometric torque extension (60°)	$p < 0.05$	$p > 0.05$
Isometric torque extension (70°)	$p > 0.05$	$p > 0.05$
Isometric torque extension (80°)	$p > 0.05$	$p > 0.05$
Isometric torque extension (90°)	$p > 0.05$	$p > 0.05$
Isometric torque extension (100°)	$p < 0.05$	$p > 0.05$
Isometric torque extension (110°)	$p > 0.05$	$p > 0.05$
Isokinetic torque flexion (30°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (60°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (90°/sec)	$p > 0.05$	$p > 0.05$
Isokinetic torque flexion (120°/sec)	$p > 0.05$	$p > 0.05$
Isokinetic torque flexion (180°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (240°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (30°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (60°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (90°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (120°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (180°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (240°/sec)	$p > 0.05$	$p > 0.05$

Chapter 4: Vit-D group

	Pre to Post immobilisation	Pre to Post2 immobilisation
Isometric torque flexion (60°)	$p < 0.05$	$p > 0.05$
Isometric torque flexion (70°)	$p > 0.05$	$p > 0.05$
Isometric torque flexion (80°)	$p > 0.05$	$p > 0.05$
Isometric torque flexion (90°)	$p < 0.05$	$p > 0.05$
Isometric torque flexion (100°)	$p > 0.05$	$p > 0.05$
Isometric torque flexion (110°)	$p > 0.05$	$p > 0.05$
Isometric torque extension (60°)	$p < 0.05$	$p > 0.05$
Isometric torque extension (70°)	$p > 0.05$	$p > 0.05$
Isometric torque extension (80°)	$p > 0.05$	$p > 0.05$
Isometric torque extension (90°)	$p < 0.05$	$p > 0.05$
Isometric torque extension (100°)	$p > 0.05$	$p > 0.05$
Isometric torque extension (110°)	$p > 0.05$	$p > 0.05$
Isokinetic torque flexion (30°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (60°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (90°/sec)	$p > 0.05$	$p > 0.05$
Isokinetic torque flexion (120°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (180°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque flexion (240°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (30°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (60°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (90°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (120°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (180°/sec)	$p < 0.05$	$p > 0.05$
Isokinetic torque extension (240°/sec)	$p > 0.05$	$p > 0.05$